JOURNAL OF AGRICULTURAL RESEARCH

VOL. XXIV WASHINGTON, D. C., JUNE 16, 1923 NO. 11

MORPHOLOGY AND HOST RELATIONS OF PUCCINIASTRUM AMERICANUM[1]

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The orange rusts have always been considered as practically the only rusts of economic importance on species of Rubus in America. *Kuehneola uredinis*, it is true, is known to cause considerable damage to fruiting canes of certain cultivated varieties of blackberries. *Pucciniastrum americanum* (Farl.) Arth. is not rare on wild raspberries but its effects on the host have been found heretofore to be altogether negligible. Brief notes dealing mostly with its relationships to the European form *P. arcticum* have appeared from time to time but no reference has been made to damage to cultivated raspberries. An outbreak of this rust, therefore, of such severity as to cause defoliation and spotting of canes of certain varieties, becomes a subject of interest.

One of the native hosts of this rust is the wild *Rubus strigosus*, and like the orange-rusts and the stemrust, Kuehneola, the Pucciniastrum will no doubt be found wherever the natural hosts may grow. The rust has been reported rarely on *R. occidentalis*, but "*R. neglectus*," a hybrid between the red and the black raspberries, seems to be rather susceptible according to Farlow (*9, p. 13*).[2] At Bell, Md., where an outbreak occurred in 1922, Mr. G. M. Darrow has gathered together from all parts of the world a number of species and horticultural varieties of Rubus and has originated many new forms in connection with his breeding work. During July it was found that the leaves of several different hybrids were dropping off prematurely, leaving the canes entirely bare below. The lower surface of the leaves was evenly covered with uredospores, giving them nearly the same appearance as blackberry leaves heavily infected with the Kuehneola. The leaves at the growing tips were sometimes only slightly affected and the attacks seemed to increase in severity from the tips downward, showing that the rust had been present for some time.

Conical peridia (Pl. 1, C), each surmounted by a corona, were easily found on leaves just being attacked, and as the spores were identical with those found on the fallen leaves there can be no question that this defoliation was being caused by what has heretofore been considered a harmless rust. From July 28 to August 4 a survey was made to ascertain which species, varieties, and hybrids were being attacked. Mr. Darrow cooperated with the author in going over these records.

[1] Accepted for publication Jan. 22, 1923.

[2] Reference is made by number (italic) to "Literature cited," pp. 893-894.

Journal of Agricultural Research.
Washington, D. C.
aew

Vol. XXIV, No. 11
June 16, 1923
Key No. G-311

43325—23——1

Certain varieties were represented by as many as 75 plants, others by only 1 or 2, and conditions for infection were perhaps more favorable on one side of the field than on the other, so that a full report on these observations would not fully represent the actual degrees of susceptibility. Among others, the following seedlings were found to be quite badly infected: Ranere × Cuthbert,[3] Cuthbert × *R. lasiostylus*, La France × Ranere, Van Fleet × Ranere, Laxtonberry × Ranere, Cuthbert, Gregg × Cuthbert, Brighton × La France, Brighton × *R. lasiostylus*, *R. coreanus* × Brighton, Shaffer seedlings, Shiebley × King, *R. leucodermis* × King, Newman 23 × *R. coreanus*, seedlings of Hailsham open pollinated, Syracuse open pollinated, Brighton selfed. The Ranere (St. Regis) was infected, but not as heavily as were the Cuthberts.

Certain hybrid seedlings were so severely infected as to result in almost complete defoliation, petioles and canes also being attacked. These very susceptible kinds include such hybrids as June × Cuthbert, Latham × Brighton, Latham × Ranere, Erskine × Ranere, *R. coreanus* × Brighton, etc.

A number of varieties in this plot were only slightly infected, but, as previously noted, the number of plants in many cases was too small to prove that they were resistant. About 50 plants each of the following forms were carefully examined at this time, but no rust was found: *Rubus Idaeus*, open pollinated seedlings of the Mahdi (which is a cross between *R. Idaeus* and a European blackberry), *R. innominatus* × Antwerp, *R. innominatus* × King, *R. innominatus* (open pollinated seedlings). A few self-pollinated Cuthbert seedlings in this field showed no rust, but many plants of this variety in a garden near by were heavily infected.

Separated from these plots by about 40 rods was a third plot, in which several plants of wild *Rubus occidentalis*, *R. strigosus*, *R. leucodermis*, King, Cuthbert, Ranere, etc., were growing. A few pustules were found on one unnamed variety. A month later the rust was very abundant on the King, June, Newman 23, Cuthbert, and some was found on *R. strigosus*, but *R. occidentalis*, *R. Idaeus* (Hailsham variety), and 5 Asiatic raspberries were not affected.

Mention has been made of finding the rust on leaf stalks and canes. The cankers on the latter are sometimes 2 or 3 inches long. A species of Gloeosporium (not anthracnose) was also common in large cankers on the varieties whose canes were most heavily attacked by the rust. It is possible that this fungus became established in the lesions first formed by the rust; at any rate the combined effect of the two fungi will be to kill the canes. The calyx and carpels of late-fruiting or everbearing varieties were sometimes badly rusted. It is certainly curious to see the golden-yellow pustules deeply embedded in the juicy red carpels. There was more rust on the fruit of a late-flowering form of *R. strigosus* than on the leaves.

A press of work prevented a satisfactory examination of species of wild and cultivated raspberries in other localities to learn how common the rust was in the vicinity. It is clear, however, that the rust had found in these hybrids very susceptible hosts.

[3] The Black Pearl, Kansas, Cumberland, Gregg, and Plum Farmer, are American horticultural varieties of black raspberry which were originated as seedlings or by selection from *Rubus occidentalis*, of which *R. leucodermis* is a western variety; the Shaffer, Columbian, and Royal Purple are of American origin, probably derived from the form *Rubus neglectus*, which is a hybrid between *R. strigosus* and *R. occidentalis*; the Cuthbert, King, Victor, Erskine, June, Latham, Ranere, and Brighton, are American horticultural varieties of red raspberries, and related to *R. strigosus*; *R. Idaeus*, the Antwerp, La France, Laxtonberry, and the Hailsham, are European red raspberries; *R. coreanus* and *R. innominatus* are native of China.

An inspection of the berries grown at the Government farm at Arlington, Va., was not made until the latter part of September. From 6 to 50 hills each of Cuthbert, King, Ranere, Antwerp, wild *Rubus occidentalis*, Kansas, Black Pearl, Cumberland, Plum Farmer, Shaffer, Columbian, and Royal Purple are grown. The Cuthbert red raspberry was the most severely attacked; much defoliation had resulted, and the remaining lower leaves were well covered with uredinia. Considerable rust was found on the Columbian, Royal Purple, and the Victor. The Ranere (St. Regis) and King varieties bore some rust, but not enough to cause any damage. No rust was found on any of the black raspberries grown here, and as *R. occidentalis* (at Bell, Md.) showed no rust it may be assumed that our black raspberries are very resistant. It is not strange to find that crosses between the black and the red varieties in many cases prove to be very susceptible. Whether the strain of the rust which made its appearance in the vicinity of Washington, D. C., is more virulent than that heretofore found on *Rubus "neglectus"* and *R. strigosus* is not known, but it is evident that many of the hybrids at Bell are more susceptible than either of the parents.

The host is occasionally erroneously identified by collectors, especially in the case of the red raspberry, which is often confused with the black raspberry. For example, in the Path. Coll., B. P. I., is found No. 2280, N. A. Uredinales, one gathering at Madison, Wis., October 6, 1906, and another at Mackinac, Mich., September 6, 1894, in which the host is given as *R. occidentalis* when it is a red raspberry. Arthur (*2, p. 190*) states that *R. occidentalis* was erroneously given as a host for *Pucciniastrum americanum* in Bull. Torrey Club (*1, p. 468*). In the same article a specimen from Algoma, Wis., was referred to *P. arcticum*, and the host is given as *R. occidentalis*. The rust is clearly of the *P. americanum* type, and the host is *R. strigosus*. Davis (*6*) has shown that *R. occidentalis* can be infected by *P. americanum* from *R. strigosus*. We have not found pustules of this rust on the wild black raspberry in the vicinity of Washington, D. C., but more careful search would very likely result successfully. The rust is undoubtedly more widely distributed than is commonly supposed. Specimens on cultivated raspberries have been referred to other species by mistake. In the Path. Coll., B. P. I., we find one packet of the rust on a red raspberry originally labeled *Lecythea Ruborum* Lev.; No. 32, Economic Fungi, Seymour and Earle, *Phragmidium Rubi-Idaei* (P) Wint. is *Pucciniastrum americanum*. One gathering of Pucciniastrum on *R. strigosus* was sent to the author as orange-rust in response to a request for red raspberry plants infected with the Gymnoconia.

THE MORPHOLOGY OF THE UREDINIUM

Farlow (*9*) recognized that the rust which he had found on a hybrid raspberry differed somewhat with respect to the shape of the peridium from *Pucciniastrum arcticum* of Europe. The peridium was more sharply conical than that of *P. arcticum*, which is said to be low and broad. He called attention to a good illustration of the peridium of the American form by Dietel in Hedwigia (*7, p. 331*) under the name *Phragmidium gracile*. Farlow proposed the variety name *americanum*, which Arthur (*1, p. 468*) later raised to specific rank. Davis (*5*) has shown that *Pucciniastrum arcticum* is very common in Wisconsin on *Rubus triflorus* and questioned at that time whether the differences in the morphology noted by Farlow may not after all be due to host differences. He is now convinced (*6*),

however, that the two forms are distinct and reports the work of one of his students who failed to infect *R. strigosus* with uredospores from *R. triflorus*, whereas *R. strigosus* and *R. occidentalis* were infected with spores from *R. strigosus*. The reader is not informed just what was done in the way of attempting to infect *R. triflorus* with spores from either host. The leaves of *R. arcticus* and *R. triflorus*, practically devoid of tomentum on the lower surface, are certainly quite different in texture from those of our common raspberries. No one would question that there may be, as we have shown, certain hosts that are more susceptible than others, and it is possible that differences in texture of tissues attacked by the rust could very well account for the differences or modifications in the form and size of sori which originally led Farlow to suggest that the form on *R. strigosus*, etc., be called *P. arcticum* var. *americanum*. The presence of *P. americanum* at Bell, Md., where pustules occurred so abundantly on leaf blades, midribs, petioles, canes, floral envelopes, and even on the fruit, offered a rare opportunity to study the morphology of the sorus.

Our sections show that there is a great variation in the form of the uredinia developing on the same plant. Some are sharply conical with coronate peridia; others are low and broad. Pustules were found to vary in size from 50 μ to 1 mm. in diameter. The peridia of uredinia developing on the under side of the leaf (Pl. 1, C) conform to the type described by Farlow. On the larger veins and especially on the petioles they tend to grow much larger and round out, sometimes becoming nearly globular (Pl. 1, B). The corona is not conspicuous in such peridia, especially where they are rather deep-seated, originating beneath three or four layers of cells.

Sori on canes are much flattened, elliptical or lenticular (Pl. 1, D, E, F, G, and 3, A). In such cases the heavily cutinized epidermis effectively prevents the protrusion of the peridium (Pl. 1, G), so that the peristomal cells and most of the peridium often disappear through disorganization. Spores are discharged through the passageway opened by the crushing and thrusting aside of the peridial and host cells above (Pl. 1, A).

Deep-seated sori are apt to occur in the calyx and fruit where the apex of the peridium seldom appears through the epidermis. Adjacent sori merge by the dissolution of side walls, large saclike cavities which are packed with spores resulting. Sori that do not lie much over two or three cells deep in the tissues beneath the epidermis are erect (Pl. 1, F), broadly elliptical, or lenticular. While individual sori on canes are large, they increase in length by coalescence (Pl. 1, G). Very frequently the vegetative hyphae mass together, crushing aside the host cells of the cortex. If these primordia lie near the forming cork layers, or lie deep down in the cortex, the uredinia will be inverted, the "peridium" being formed on the inner side (toward the center of the cane) (Pl. 1, D, E).

Two sori may originate from the same primordium, one having a peridium on the side toward the epidermis, the other on the side next to the cork cambium, which lies quite deeply embedded in the cortex. The sori shown in Plate 1, F, originate from separate primordia; the one above is erect, the other inverted.

ORIGIN OF THE SORUS AND ITS PERIDIUM.

The origin of the sorus is most easily studied from sections of the rust as it occurs on leaf stalks. The vegetative hyphae are not well provided with a granular or stainable cytoplasm, but the cells giving rise to the

primordium increase in size and their contents become granular and stainable and the nuclei show very distinctly (Pl. 2, A). Cells become arranged side by side in a compact plectenchyma. The terminal cells which eventually constitute the peridium elongate rapidly, lose their granular cytoplasm and nuclei by disorganization, and act as buffer cells to push aside and crush the host cells above. In Plate 2, B, two or three terminal cells at each end of the section are more heavily shaded in the drawing to indicate that they were more deeply stained than the others; the final divisions by which peridial cells arise will soon take place. In this way the sorus continues to increase in breadth for some time. In the formation of the teleutospores of species of Gymnosporangium (*8*) terminal cells of a sorus primordium function purely as buffer cells and entirely disappear as the sorus matures. It is the subterminal cells from which the teleutospore buds are formed. This is not the case in *Pucciniastrum americanum* on petioles, where the buffer peridium persists more or less and is usually recognizable in an old sorus, while the subterminal cells disappear.

Later the author will consider spore formation as found in *P. agrimoniae*, the species studied by Ludwig and Rees, but if Plate 2, A and B, of this paper is compared with their figure 1 in Plate VIII it will be seen that the only essential difference is in the layer of somewhat shorter or more flattened subterminal cells which the author found in various stages of disorganization, indicated by the way these cells take the orange G. In their figure the subterminal cells in the four central rows are not as thick as those below them. Sections presenting such features as are shown in Plate 5, A, of this paper certainly can be interpreted to mean that the layer of cells, b, which extends across the sorus, is composed of active basal cells, since these cells take the gentian violet stain rather deeply, while the cells of the "hyphal plate" and vegetative cells below take scarcely any stain. The cells s take the orange and safranin and certainly appear to be degenerating. The cells u will develop into spores. Toward the center of this sorus space has been formed and the intercalary cell below the spore is elongating into a stock as it disorganizes. If this method of spore formation were continued, the uredospores would be borne in chains, and essentially there is very little difference between the two methods. In very compact deep-seated primordia, conditions may be such as to necessitate the formation of the first spores in this way. A greater upward thrust against the overlying tissue would result if the basal cell elongates as a whole or at its upper end in preparation for division. In Plate 5, B, are shown the first spores formed in a sorus where the pressure was soon relieved at the center by a break in the overlying tissues. Large wedge-shaped uredospores with perfectly definite stalks can be seen. Nearer the margin of this sorus there was still considerable resistance at the left and above, so that the spore initial buds developing from the basal cells, b, are pushing out to the right where the tissue was less compact. The relationship of peridial cells, p, intercalary cells, i, and basal cells, b, is very evident in sections of this sorus. Further evidence that pedicellate spores occur in more mature sori is scarcely necessary. At the right in Plate 5, C, is shown a perfectly typical pedicellate spore. At the left three adjacent basal cells are budding; nuclear division is occurring in the cell at the middle.

If the tangled mass of hyphae constituting the sorus primordium is exceptionally large the development of a clear-cut peridium is more or

less interfered with, and uredospores often appear to be formed from almost any cell of the vertically oriented hyphae. The dark area in the sorus shown in Plate 1, G, indicates where disorganization of abnormal spores or of a portion of the primordium tissue is occurring, so that space will be provided and the host tissue above ruptured. More detailed studies of the process involved in spore production in such atypical sori should be made in order to determine to what extent these apparent variations from true form are really the results of abnormal conditions.

The "hyphal plate" layer mentioned by Ludwig and Rees is commonly present along the base of the sorus in *P. americanum* and seems to be almost continuous with the peridium, as shown in Plate 1, B. The origin of this tissue is perfectly clear. The columns or chains of cells constituting the plectenchymatous primordium are the much enlarged, vertically oriented branches of the ordinary hyphae which originally formed a small tangled mass at the commencement of the uredinium. These hyphae can not be distinguished from the purely vegetative hyphae by the size, form, or staining properties of their cells. Now, the sorus increases in breadth simply by the lateral extension of branches from this undifferentiated tissue. Certain branches grow out vertically and parallel the other columns of cells and form peridial, intercalary, and basal cells in regular order, so that there are bound to be platelike layers of cells, especially along the base at the margins, which connect up with the peridium.

In order to secure further proof of the homology of intercalary cells and uredospore stalks, the author gathered and fixed material showing various stages in the development of uredinia of *P. agrimoniae* and *P. hydrangeae* on November 2 at Occoquan, Va. A study of sections of *P. agrimoniae* showed that Ludwig and Rees have given a good figure of a young sorus. There is ample evidence, on the other hand, proving that intercalary cells which degenerate are cut off below the peridium (Pl. 5, D, E) and that such cells are also formed with at least the first uredospores (Pl. 5, F). Many cases were observed where there could be no doubt that the basal cell forms one or more buds, spore initials (Pl. 5, G), each of which in turn divides to form a spore supported by a stalk. The stalk may disappear without much or any elongation. A few other stages in spore formation are figured here showing that *P. agrimoniae* does not differ materially in the organization of its uredinium from *P. americanum*.

Much of the material of *P. hydrangeae* was probably gathered too late in the season, as most of the sori sectioned had aborted or undergone degeneration after having developed the chains of cells constituting the primordium. Such stages are, in this condition (Pl. 5, J), apt to be misleading. In a few cases the uredinia appeared to be healthy and normal. Some evidence was found showing that the intercalary cells are cut off as the peridium is formed (Pl. 5, K). Certain irregularities in the structure of this tissue need further study. One could find stages where the first spores were being formed, showing that the uredospores originate as a result of the cutting off of a spore initial from the basal cell as a whole and not as a bud. In this species the cell supporting the spore must be considered the homolog of a true stalk cell or pedicel. Young spores supported by intercalary cells are shown in Plate 5, H.

Plate 3, A, B, shows more clearly that the peridium is composed of the terminal cells of the sorus primordium. The subterminals take the

orange stain more deeply than the others, therefore this layer is easily recognized in sections. Disorganization, followed by collapse, occurs, so that these interstitial cells are represented finally by more lines of degeneration products.

METHODS OF SPORE FORMATION

The formation of uredospores in Puccinia from a definite layer of basal cells which send out buds (spore initials) is well understood. Nuclear division is followed by cell division, cutting off from the spore initial the stalk cell below and the spore above. Such spores are thus not borne in chains, even though the basal cells may continue to bud and give rise to a number of spores.

Liro (*10*, *p. 490*, *492*, *493*) states that the uredospores of *Melampsorella cerastii* and *M. jeurichii* are borne in caeomalike sori, that is, in chains. Magnus (*12*), while confirming Liro regarding the first species, disagrees with him as to the second, figuring stalked spores in a sorus of *M. jeurichii*, and further holds that it would be impossible for a spore to be formed out of such stalk cells. He creates the new genus Milesina, especially characterized by stalked uredospores, leaving in Melampsorella *M. cerastii*, which he and Liro agree has catenulate spores.

Ludwig and Rees (*11*), who made a study of the uredinium in *P. agrimoniae*, find that the apical cells of the primordium elongate and their contents stain less deeply. The terminal cells become the first peridial cells, and the other cells of the chains are the uredospores, no intercalary cells being formed. These authors further state that the spore chains arise from a layer of basal cells just above a "hyphal plate layer," which is a tissue extending across the base of the sorus and connecting at the margins with the peridium. Stages in spore formation are not shown, although they follow Magnus in their explanation of the probable method. Since intercalary cells were not found, they suggest that the peridium is formed in a way analogous to that in the ordinary aecidium. They also suggest that the genera now included in the Pucciniastratae may be separated into two subgroups, the fern rusts having pedicellate spores in one group, and Pucciniastrum, Melampsorella, Melampsoridium, characterized by spores borne in chains with each chain maturing but one spore at a time, in the other.

The writer has not made an extensive study of the order of cell division in spore formation in *P. americanum*, but there can be no question that the uredospores are frequently borne singly on pedicels. On the other hand, if the very important fact that intercalary cells are present is disregarded, there is considerable evidence showing that the first spores, especially in deep-seated sori on petioles and canes, are borne somewhat as Magnus describes for Melampsorella. In other words, in *P. americanum* the first spore initials arise as the result of tangential divisions of basal cells and not by budding. Any claim that the upper daughter cell may sometimes become a spore at once, the sister cell remaining as a basal cell, would be difficult to disprove.

FORMATION OF THE OSTIOLE

The formation of the ostiole and the peristomal cells in *P. americanum* can be followed very readily by studying sections of sori which are not very deeply seated. About the time the first spores appear in the cavity beneath the central part of the buffer peridium, it will be seen that a few

cells at the center or apex are beginning to disorganize and collapse at their lower ends (Pl. 1, A, and 5, B). As the peridium is lifted these central cells sustain considerable pressure from the sides so that they eventually become distorted, compressed below, enlarged above, and are finally thrust somewhat out of place as they break through the epidermis. The spinelike thickenings which are being formed during the rupturing processes may in some way facilitate the final dislodgment of the peristomal cells in older sori. The cell ps in Plate 4, E, is slightly more than half the length of that of the adjacent peridial cell, p. Originally these two cells were the same length; therefore, the difference seen later is due, not to the thickening of the wall of the lower end, as thought by Farlow, but to disorganization which is not confined to the peristomal cells alone. It is interesting to see that while the lower end of such cells is becoming disorganized and distorted the cytoplasm and nuclei in the upper part are still active, holding the gentian violet stain to the last or until the spines are formed.

It is now a well-recognized fact that peridial cells of an aecium are the homologs of aeciospores, being merely the specialized terminal cells in the chain of spores. The figures in this paper suggest that these homologies can be carried over to the uredinium of Pucciniastrum, where the peridial cell and intercalary cell below are comparable to the uredospore and its stalk, which supports the theory first advanced by Sappin-Trouffy (*13, p. 86*) and further defended by Christman (*4*).

Stalked spores arising from budded basal cells were not found in the limited study of *P. hydrangeae* made by the author. Such types as are shown in Plate 5, I, suggest that in older sori the intercalary cell may elongate to take the form of a stalk and that the basal cell may bud the same as we find in the other species.

It is a certainty that spores are sometimes formed atypically in *P. americanum*, so the author has hesitated to interpret the figures in Plate 3, which show (at the right) spores without stalks or supporting intercalary cells. The author has relied on the positive evidence offered by finding intercalary cells and pedicellate spores in just such sori, rather than on such negative evidence as these figures might be interpreted to afford. In the orderly process of spore formation in uredinia there is a hymenial layer of basal cells from which spore initials regularly arise, but all sorts of abnormalities are liable to occur here as in other fungi, so that a spore might be formed from almost any cell, peridial, intercalary, or basal. Every well-nourished cell in the primordium is potentially sporogenous, but usually one of the daughter cells of the spore initial is in effect sacrificed, thus providing additional food for the one that is to become the spore; the other, although incidentally becoming a disjuncting intercalary cell or an elongated stalk, finally degenerates.[4]

Magnus' figure 7, Plate XIV (*12*), in support of his view is not convincing. He may have overlooked the stalk cells or their homologs, intercalary cells, as he did the peridial cells in Hyalopsora which Bartholomew (*3*) shows very plainly to be present in this genus. Ludwig

[4] L. Kursanov, in a recent paper which has just come to hand (Recherches morphologiques et cytologiques sur les Urédinées. Bul. Soc. Nat. Moscow, v. 31, p. 1–129, pl. 1–4, 25 fig. Oct., 1922), traces the development of terminal sterile cells in the uredo sori of *Puccinia allii, Triphragmium ulmariae, Uredo (Pucciniastrum) pirolae*, and *Hyalopsora polypodii-dryopteridis*. He figures intercalary cells directly beneath the sterile terminal cells in the last two species and states that they arise by the division of the subterminal cells. This may very well be their method of origin in the species of Pucciniastrum which the writer has under discussion, but it would not affect their homology. Instances of the cutting off of the intercalary cell from the upper part of the aecidiospore initial are well known. Kursanov has not followed fully the development of the uredo spores in *Pucciniastrum pirolae*, so that the reader is left in doubt as to whether they are provided with true stalk cells or not.

and Rees, as noted, state that the uredospores of *Pucciniastrum agrimoniae* are borne in chains and that the terminal cells of the chains elongate to become the peridial cells, the other cells developing one by one into uredospores as described by Magnus for Melampsorella. These authors did not find intercalary cells either in the spore chains or below the peridial cells. If this were true, the peridium would be formed in a manner analogous to that of the aecidium, as they pointed out.

Some of the strongest hints as to the trend in evolution are suggested by what it is thought are atypical processes; but if in addition to the method of spore formation which the author has observed in Pucciniastrum it should be rarely found that every cell below the peridium in the cell chains develops into a uredospore, as claimed by Ludwig and Rees, the doctrine of homologies would be weakened. The buffer cell in the telium of Gymnosporangium may be the homolog of the peridial cell in Puccinastrum; this depends on just how the first intercalary cell in the latter is cut off. It is not the homolog of the peridial cell of the ordinary aecidium, but rather it is comparable to the hymenial basal cell or its sister peridial initial. If the uredospores of *Melampsorella cerastii* are always borne in chains, and those of Hyalopsora are borne singly on pedicels, then the species of Pucciniastrum form a good series of connecting links, *P. hydrangeae* being more nearly like Melampsorella, especially if we should find intercalary cells in the latter.

The complete life history of *P. americanum* is unknown. Three other species of the genus have their aecial stage on Tsuga and two on Abies. The occurrence of our rust on canes of the current season suggests that the fungus may live through the winter, thus obviating the necessity for the alternate aecial stage in certain localities. No explanation has been offered to account for the presence of the deep-seated, inverted sori on canes, which are very common. It is possible that the inversion may facilitate spore discharge the following spring as the cortical parenchyma peels off, exposing the cork tissue below. Such inverted sori were not found on leaves or petioles.

It has been shown that the form and size of sori vary greatly, depending upon what tissue they are found, indicating that *P. arcticum* and *P. americanum* are not distinct species. In the event that it can be shown that *Rubus triflorus* and *R. arcticus* can not be infected with spores from *R. strigosus* and related forms, distinct names for the strains or biologic forms might still be desirable.

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PLATE 1 [a]

Pucciniastrum americanum (Farl.) Arth. on June × Cuthbert Red Raspberry

A.—Section through the center of a young uredinium on leaf stalk. Four layers of host cells lie above the sorus. Cells next to the sorus have been crushed by the expansion of the buffer tissue. Disorganization of cells at the center. Uredospores formed irregularly, intercalary cell replaces stalk.

B.—Vertical section through a mature sorus on a leaf stalk. Only two layers of host cells above. The buffer tissue has been flattened by the pressure from within and will persist more or less as a peridium, although the cells at the center are fully collapsed and will soon disappear. No corona would have been formed. Uredospores stalked.

C.—Vertical section of sorus on lower side of leaf. One cell of the corona showing.

D.—Inverted broadly elliptical sorus on young cane. "Peridium" or buffer tissue facing the cork cambium, which is deep-seated; e, epidermis; ck, cork layers.

E.—Inverted sorus still more deeply seated. Buffer tissue directly against the cork.

F.—Two lenticular sori on a cane. The upper one lies just beneath the epidermis and is erect; the lower is inverted.

G.—Erect double sorus on large cane. Peridium of the part at the left has entirely disappeared, cavity filled with spores. At the right peridium still evident although disorganizing. Degeneration of tissue at the center of the sorus (dark area), normal spore production below. Note the position of the epidermis above the sorus and of the cork cambium below it; e, epidermis, cp, cortical parenchyma, ck, cork.

B to F, photographed with Leitz 8 mm. lens and No. 10 oc.; A, 4 mm., 6 oc.; G, 16 mm., 10 oc.

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[a] The photographs shown in Pl. 1, and all the sections which were studied in the preparation of this paper were made by Miss Ruth Colvin.

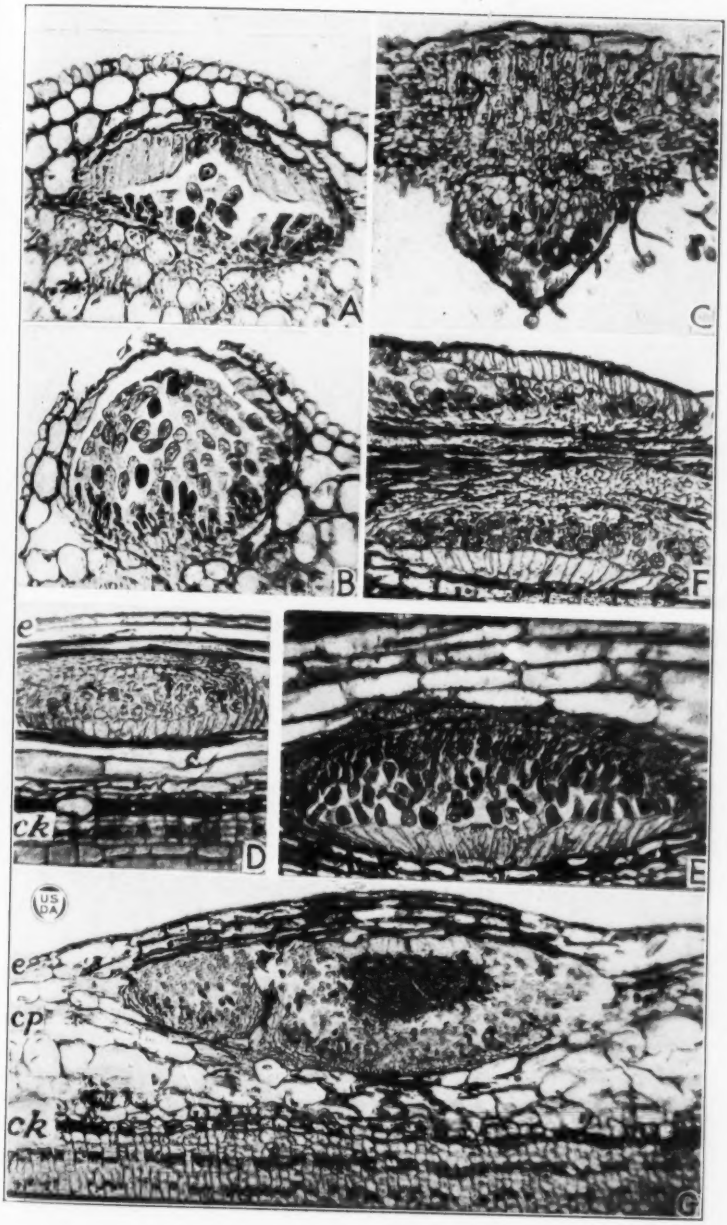
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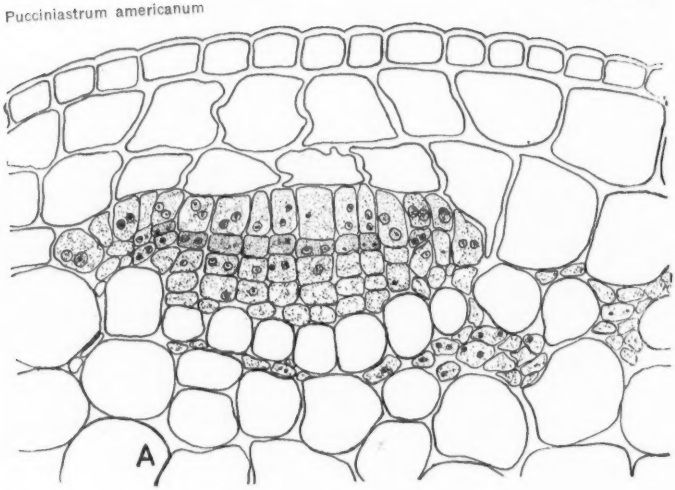
PLATE 2

Pucciniastrum americanum



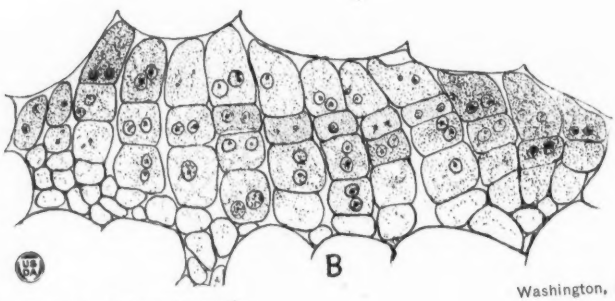


PLATE 2

A.—Primordium of sorus on leaf stalk of raspberry, showing its position in the host tissues. Peridial or buffer cells, each accompanied by a disorganizing intercalary cell, at the center of the sorus. Cell division by which intercalary cells are formed has not occurred at the margins.

B.—Primordium of sorus on petiole of raspberry. Terminal cells elongating, intercalary cells disorganizing. Cells at the margin just before division.

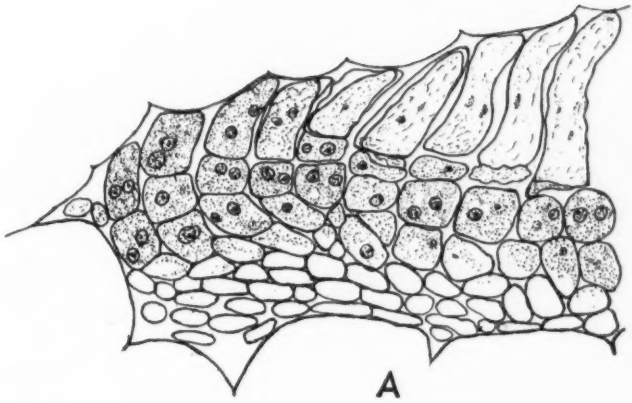
PLATE 3

A.—Part of section of a young sorus on leaf stalk. Peridial cells fully elongated at the right, contents disappearing, intercalary cells collapsing.

B.—Part of section of a small and rather flat sorus on a cane. Buffer tissue of erect cells; intercalary cells in all stages of disorganization. Five uredospores at right center.

Pucciniastrum americanum

PLATE 3



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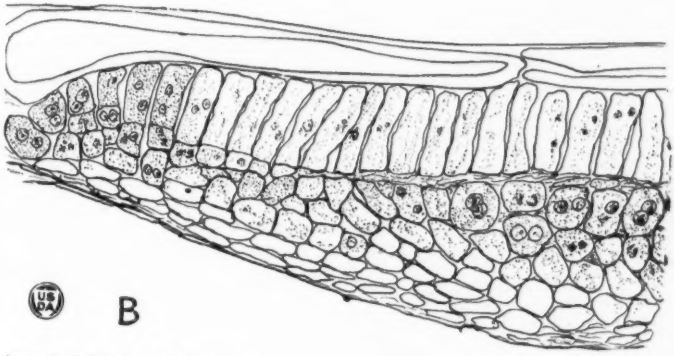

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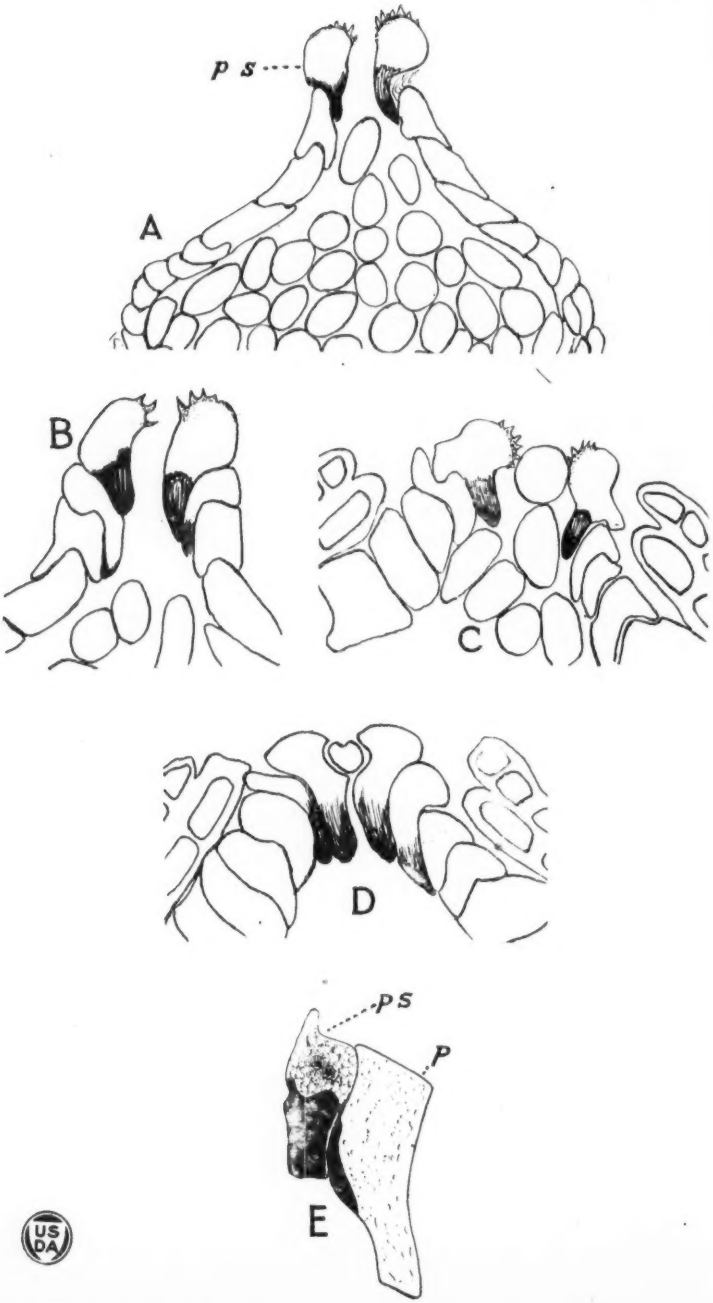
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PLATE 4

The origin of peristomal cells in *P. americanum*. Deep shading of the peristomal cells, PS, indicates disorganization; P, peridial cell. (See text for further explanation.)

PLATE 5

P. americanum

A.—Columns of cells in a young, compact sorus. Uredospores arise by division of spore initial cells: p, peridial cell; i, intercalary cell; u, uredospore; s, stalk; b, basal cell.

B.—Formation of stalked spores in a sorus in which space was provided by the early rupture of overlying tissues. Spore initial buds are arising from basal cells (at the left) so that the first spores will be borne on stalks.

C.—Uredospores formed from budding basal cells. Nuclear division occurring in the bud from the second basal cell.

P. agrimoniae

D.—Row of cells at margin of sorus, wedge-shaped intercalary cell below the peridial cell p.

E.—Fully elongated peridial cells supported by intercalary cells beneath which are normal basal cells, not spores.

F.—Typical spore initial bud si, from basal cell (right); cell chain consisting of young uredospore, intercalary cell, basal cell, and "hyphal plate" cell; stalked spores.

G.—Short stalk cells. Whether we find two spores or only one connected with the basal cell may depend on the way the cell is sectioned.

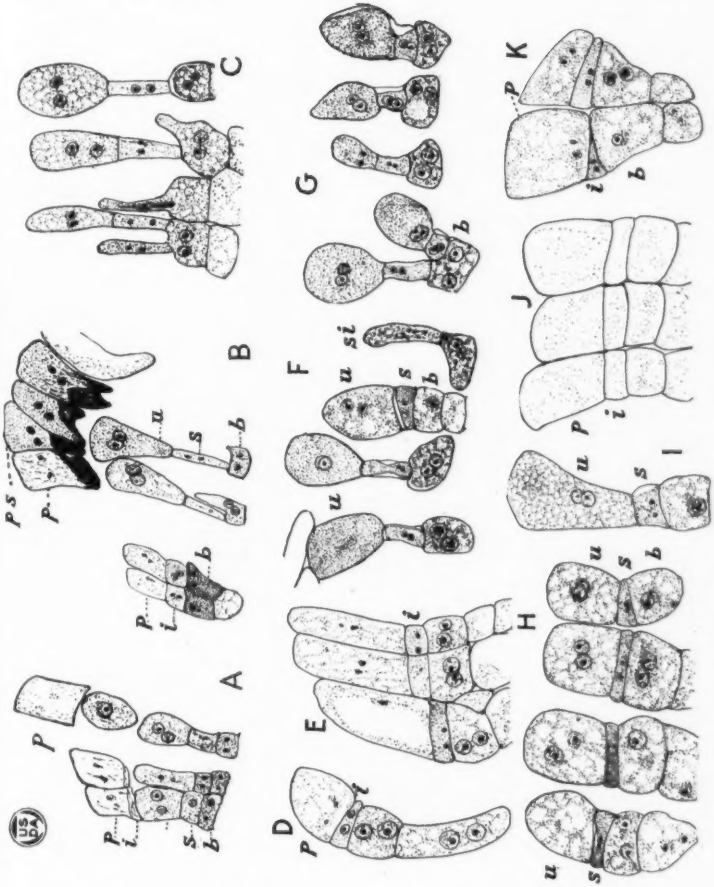
P. hydrangeae

H.—Four young uredospores, u, each supported by an intercalary cell.

I.—Intercalary cell elongating somewhat.

J.—Chains of cells in an aborted sorus. Intercalary cells beneath the peridium.

K.—Cell chains at the margin of a normal young sorus; wedge-shaped intercalary cells degenerating.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WATERY-ROT OF TOMATO FRUITS[1]

A PHYSIOLOGICAL FORM OF OOSPORA LACTIS; EFFECT ON THE HOST; PENETRATION OF THE CELL WALLS BY ENZYMIC ACTION

By FRED J. PRITCHARD, *Physiologist*, and W. S. PORTE, *Scientific Assistant, Office of Cotton, Truck, and Forage Crop Disease Investigations, Bureau of Plant Industry, United States Department of Agriculture*

INTRODUCTION

A new rot of tomato fruits closely resembling the rot caused by *Bacillus carotovorus* has frequently been found in shipments of southern grown tomatoes and sent to the Office of Cotton, Truck, and Forage Crop Disease Investigations by the Bureau of Markets' inspectors since the spring of 1921. It is also prevalent in the vicinity of Arlington, Va., and Washington, D. C.

This rot is characterized by the extremely watery appearance and condition of the affected tissues, by the absence of any other discoloration, and by the occasional oozing of water from the surface—features by which it can readily be distinguished from other fungous softrots. The rotted areas usually develop in the form of sectors extending from the stem scar toward the blossom end. In very humid air they are partly covered with a white velvety to granular fungous growth, but under average atmospheric conditions this is absent. The illustration shown in Plate 4, A, is fairly typical of this rot except for the low point of origin and the presence of a surface growth.

That this rot has frequently been mistaken for the rot caused by *Bacillus carotovorus* is quite likely, as the two are similar in macroscopic appearance. However, it differs from the bacterial rot in rapidity of development; in fact, fruits kept in the laboratory 10 days after this rot had made considerable progress were not completely softened, while those infected by *B. carotovorus* usually collapsed in about 3 days.

As an examination of the affected fruits obtained from the Bureau of Markets always disclosed the presence of an Oospora, experiments were made to determine the relation of this fungus to the disease.

INVESTIGATION

MATERIAL AND METHODS

The Oospora used in the inoculations was grown chiefly on carrot agar, as it grew better on it than on most other kinds of media. It grew well also on turgid raw carrots kept in a moist atmosphere, producing a distinct rot (Pl. 4, C), but this material was used only for morphological comparisons.

In the inoculation work, green, ripe, and partly ripe tomato fruits free from blemishes were submerged for 30 minutes in a 1:1000 aqueous solution of bichlorid of mercury, washed in distilled water, and inoculated with a pure culture of the Oospora obtained from the rotted fruits.

[1] Accepted for publication Jan. 22, 1923.

This treatment caused some discoloration of the fruits but no softening. It had no visible effect on susceptibility to infection by this fungus, for fruits treated with weak solutions of bichlorid of mercury or formaldehyde as well as fruits not treated were quite as readily infected.

Controls were liberally used in all the experiments. Both the controls and the inoculated fruits were usually kept in closed glass chambers to prevent contamination from the air, but inoculated fruits kept in open dishes were quite as readily infected.

The decomposition of the cell constituents was observed on roots of carrots and on green tomato fruits. Sections of carrot roots, chiefly from the heart, and of green tomato fruits, 250 and 500 μ thick, respectively, and free-hand sections of varying thickness were used as fresh material. Pieces of green tomato fruits from spots 2 days old were treated with Flemming's medium killing and fixing solution, embedded in paraffin, and sectioned and stained for the study of fixed material.

The cultures were made as described by Brown (*3*)[3] on thin layers of media (carrot decoction, and beef bouillon + 2½ per cent glucose) in small flasks or Petri dishes, inoculated with an optimal quantity of spores, and kept at a temperature of 22° to 26° C.

The action of the enzyms on the host cells was determined by means of the live organism, the ground-dried organism, the extract from the ground-dried organism, the filtrate from cultures, and the alcoholic precipitate from the filtrate.

The organism was separated from the culture medium by passing the liquid through a double layer of Whatman's No. 50 filter paper. A few small cells passed through the filter but showed no signs of germination during the experiment.

The enzymic material was precipitated by adding 4 volumes of 95 per cent alcohol to each volume of the filtrate. The liquid was then filtered and the precipitate washed in 95 per cent alcohol, dried in a warm air current, dissolved in a very small quantity of distilled water, and used immediately.

The ground-dried organism was used in aqueous suspension. The extract of the same material was prepared by soaking the powdered fungus in water for 24 hours.

The decomposition experiments were run for 24 hours at temperatures ranging from 24° to 45° C. Growth of microorganisms was prevented by adding chloroform or toluene to the liquid in the proportion of 10 to 25 per cent, but duplicate experiments in which no antiseptic was used were also made. Chloroform and toluene inhibited growth without apparently hindering the action of the enzym.

RESULTS OF INOCULATION

The results obtained with both pricked and unpricked fruits are summarized in Table I.

Of the 277 pricked fruits inoculated, 208, or 75 per cent, became infected. The ripe fruits seemed to be somewhat more susceptible than the green fruits, but both were easily infected through punctures. The infections of unpricked fruits took place only through the stem scar. This was frequently observed in preliminary experiments not recorded. Moreover, it appears to be the cause of the position of the rotted areas of this type on most shipped fruits.

[3] Reference is made by number (italic) to "Literature cited," p. 905.

TABLE I.—*Results of inoculating tomato fruits with the Oospora associated with watery-rot*

	Green.	Ripening.	Ripe.	Total.	Fruits inoculated.				Fruits infected.			
					Green.		Ripening.		Ripe.		Total.	
					Number.	Per cent.	Number.	Per cent.	Number.	Per cent.	Number.	Per cent.
Pricked.....	160	90	27	277	118	74	64	71	26	96	208	75
Unpricked.....	16	7	5	28	0	0	0	0	[a]2	0	0	0

[a] Infected through the stem scar.

Although these infections were obtained with an apparently pure culture of Oospora, the possibility of contamination by *Bacillus carotovorus* was not overlooked. The cultures were repeatedly plated and examined microscopically for the presence of bacteria but no evidence of bacterial contamination was found in either the plates or microscopic mounts. Moreover, the fungus pricked into halved potato tubers caused only a slight superficial growth, even when allowed to stand for a considerable period of time, while *B. carotovorus* produced a very decided rot (Pl. 4, B). The infections of the inoculated tomato fruits were therefore unquestionably caused by the fungus.

The stems and leaves of 15 vigorous tomato seedlings about 4 inches tall were thoroughly sprayed with the Oospora spores and kept in a moist chamber 60 to 72 hours but no infections developed. Ten similar tomato seedlings were thoroughly pricked in stems and leaves and smeared with the spores of Oospora, but the fungus was unable to invade the tissues. It therefore seems to have little if any parasitic action on tomato plants.

MORPHOLOGY OF THE FUNGUS

The Oospora causing watery rot of tomato fruits consists of a hyaline, septate mycelium with granular contents and numerous short branches arising near the septa at an angle of about 45° from the main filament (Pl. 3, A) and other longer branches of a two-or-three-forked type (Pl. 3, B–D).

The hyphae vary from 2.5 to 7.5 μ in diameter. The younger branches are narrower than the main filament but the taper is very gradual.

Reproduction is accomplished by the breaking up of the hyphae into their cells which serve as spores. The branches shown in Plate 3, A, divide into numerous short cells which round at the ends (Pl. 3, E, F, H) and separate. The rounding appears to begin in the apical cells (Pl. 3, G), but occurs in all cells of a branch almost simultaneously. At a certain stage of their development these cells appear to be chains of spores arising from the main part of the mycelium (Pl. 3, E, F), but by the time they lose their coherence, or shortly afterward, the main filament itself breaks up by cell division and separation into numerous cells of different lengths, which round at the ends, and when short are often indistinguishable from those formed from the lateral branches. They are hyaline and granular and are capable of germinating immediately.

The transverse diameter of these sporelike cells (oidia), which for convenience will be referred to hereafter as spores, varies from 2.5 to 7.5 μ; the length from 3.2 to 40 μ and in some cases even to more than 60 μ.

Germination of the spores may start from the end (Pl. 3, I, L), from the side (Pl. 3, J, K), or from a corner (Pl. 3, I, M). Development from a corner of the cell is quite similar in point of origin to the development of the branches shown in Plate 3, A.

COMPARISON WITH OOSPORA LACTIS

The Oospora obtained from rotted tomato fruits shipped from the Gulf States was compared morphologically with the *Oospora lactis* that commonly grows on the surface of tissues in the cracks of ripe tomatoes; with two cultures of *O. lactis* received from Dr. Charles Thom, one isolated by him from pickle scum, the other sent to him from Germany; and with an Oospora isolated from green tomato fruits affected by watery-rot at the Government Experimental Farm, Arlington, Va. The two Oosporas causing watery rot and the one from Germany did not always break up into their cells so readily and completely as those from the cracks of ripe tomatoes and from pickle scum, but this habit varied with the age of the culture and with the kind of culture medium used. Aside from this difference, which was not constant, and slight differences in quantity of mycelial growth, there were no peculiarities, except in parasitism, by which one form could be distinguished from the others. The Oospora received from the Gulf States and the one obtained from green tomato fruits at the Government Experimental Farm reproduced the watery-rot in a large percentage of the fruits inoculated, but the others appeared to have no such parasitic action on tomato fruits. It would seem, therefore, that the Oospora causing watery-rot of tomato fruits, both in the Gulf States and at the Government Experimental Farm, is a physiological form of *O. lactis*. Consequently we have given it the trinomial *O. lactis parasitica*, form phys., to distinguish it from the parent species. As it is indistinguishable morphologically from *O. lactis*, it needs no further description.

It would be interesting to know how this form compares with the 9 varieties of *Oospora lactis* that Schnell (*8*) grew on sliced potato tubers, but as he made no inoculations on tomato fruits an accurate comparison of their parasitism is impossible. Six of the 9 varieties grown on sliced potatoes, 4 of which grew also on sliced cucumbers and 2 on plums, produced a discoloration of the potato tissues which would distinguish them from the form isolated from green tomatoes. Two of the others produced slimy colonies on sliced potatoes—a character not obtained with the watery-rot fungus. The remaining strain, viz, Oid. l. 557, made a feeble growth on potato tubers, agreeing in this respect with the one isolated from tomato fruits affected by watery-rot, but this has no significance with reference to its parasitism on tomato fruits.

TEMPERATURE RELATIONS

The effect of temperature on growth and infection by *Oospora lactis parasitica* is shown in Table II.

The minimum temperature obtained for germination of the spores, for growth of the mycelium, and for infection of pricked tomato fruits was approximately 2° C., the optimum temperature 30°, and the maximum 38.5°, except for infection of fruits, which was 37.5°. As there was a difference of 1° to 2° between the temperatures of adjoining chambers and some fluctuation within each chamber, these temperatures are only approximately correct.

TABLE II.—*Relation of temperature to growth and infection by Oospora lactis parasitica*

Temperature.	Growth on culture media.[a]	Infection of pricked tomato fruits.	Germination of spores.
	°C.	°C.	°C.
Maximum	38.5	37.5	38.5
Optimum	30.0	30.0	30.0
Minimum [b]	2.0	2.0	2.0

[a] Carrot agar and glucose agar.
[b] The experiments on the minimum temperature were run about a month.

EFFECT ON THE HOST

ACTION ON THE CUTICLE

It was shown in the inoculation experiments described above that this fungus is unable to infect uninjured tomato fruits except through the stem scar or other similar areas not covered by the cuticle. This is also illustrated in Plate 1, A. The fungus lay in masses on the surface of the fruit but was unable to penetrate it. However, when once inside the fruit it invaded the epidermal cells quite readily.

ACTION ON THE PROTOPLASM

Invasion of the host cells is soon followed by a gradual consumption of their protoplasmic contents. Some of the steps in this process are illustrated in Plate 1, in which B and C show an early stage, D a medium early stage, and E–J late stages. This action of the fungus on the protoplasm of the invaded cells is quite evident soon after the penetration of the wall, as it causes dissolution of the protoplasm in advance of the growing tip (Pl. 3, R). These transparent areas, or digestion vacuoles immediately surrounding the filaments, are shown more fully in Plate 1, B–D, and Plate 3, U. It would seem from these figures that the proteolytic enzym secreted by the fungus acts chiefly in the region of the growing tip. There was apparently no preference for the nucleus, as it often remained intact after most of the other cell contents had disappeared.

Quite different results were obtained with the ground dried organism, the filtrate, and the alcoholic precipitate from the filtrate. Repeated experiments with these substances produced no visible effects on the protoplasm. The failure to obtain a proteolytic action with any of these substances may be due to a rapid deterioration of the enzym, to inhibitors, or to lack of suitable technic.

ACTION ON THE MIDDLE LAMELLA

The growing fungus, as shown in Plate 1, dissolves the middle lamella slowly, causing the loss of cell coherence. This loss of coherence and the breaking up of the fungus filaments into their individual cells produce the extremely watery consistency which distinguishes this rot from other softrots in which the fungus filaments remain intact and hold the host cells together. A similar action was produced on the middle lamella by the ground dried organism, the extract from the ground dried organism, the filtrate, and the alcoholic precipitate from the filtrate of cultures 2 to 3 days old, but this activity diminished as the age of the culture

increased. In fact, the alcoholic precipitate from cultures 6 days old and the filtrate from cultures 21 days old produced no visible effect on the middle lamella. This deterioration or inhibition of the action of the pectinase from age is quite different from the rapid action obtained with pectinase from cultures of *Bacillus carotovorus* 21 days old.

ACTION ON THE CELLULOSE

The passage of this fungus through the cell walls is shown by the drawings in Plate 3, N–T, and by the intracellular filaments in the photomicrographs of Plate 1. The drawings in Plate 3, N–T, were made from partly destroyed cells of a disintegrating area of the fruit. Only the tips of growing hyphae are shown passing through the somewhat wavy and more or less separated walls. The penetration of the cell walls of normal cells by young germ tubes was also observed by means of the microscope. The more important details of this process will be described later.

No visible effect was made on the walls by the filtrate, by the alcoholic precipitate from the filtrate, nor by the mycelium in the presence of sufficient chloroform or toluene to inhibit its growth. Moreover, bits of filter paper placed in fresh cultures of the fungus and allowed to remain there for 10 days to 2 weeks did not disintegrate. The fungus filaments passed between the fibers, causing the paper to tear apart somewhat more readily after the breaking up of the hyphae than similar bits of paper kept in distilled water, but microscopic examination of the fibers failed to reveal any corroding effects. There was apparently no chemical action on the filter paper.

It might seem from the foregoing facts that pressure rather than enzymic action enables the fungus to penetrate the cell walls as described for *Pythium debaryanum* by Hawkins and Harvey (7), but further observations do not substantiate this means of penetration for Oospora lactis.

Before a fungous filament can penetrate a cell wall by means of pressure, it must attach itself to the wall, or, if in a cell, to the protoplasm in order to prevent pushing itself away from the wall as it elongates. Spores of this fungus germinated either in water or in culture solution do not attach themselves to the slide or the cover slip. Moreover, when germinated in cells of tomato fruit tissue they do not adhere to the wall or the protoplasm. When the tip of such a sporeling comes into contact with the wall, its more or less continuous growth in length usually pushes it aside, which causes it to slide along the wall. Not infrequently the position of the whole filament is thus changed, as well as the position of other sporelings lying in contact with it. In fact, such a filament may even shift its position in such a way as to remove its tip some little distance from the wall. Such short filaments go through the walls more easily at the corners of the cell because there is less chance to slide along the wall. When a filament has passed through a wall it pierces other walls more rapidly because the anchorage thus obtained holds the growing tip against a single point better than does the free spore end of a germ tube that has no anchorage.

The phenomena accompanying the penetration of a cell wall by a germ tube of this fungus throw some light on the means by which it is accomplished. By placing spores of the fungus on the top of thin sections of tomato fruit tissue mounted and covered on a glass slide and furnished with a constant supply of water, the growth activities of the

spores that settle in the cells as well as the penetration of the wall and the effects resulting from it are easily observed by means of the microscope. Some short germ tubes lying near a wall and approaching it perpendicularly go directly through it without the use of any support or anchorage to increase their pressure. The opening made in the first half of the wall is a hole, not a basin or general depression such as would be produced by pressures, although the second half of the wall, i. e., the wall of the cell undergoing invasion, is sometimes pushed back. Whenever an invading filament completely fills the hole in the first half of the wall it attains a certain amount of anchorage which no doubt enables it to make some use of its growth pressure. This causes the second half of the wall to bend back before the filament has passed through it (Pl. 3, P, T), but it is not essential to the penetration. Moreover, this bending is usually absent, because the hole made in the first half of the wall, as shown by focusing sharply with the microscope, is usually a little larger than the filament (Pl. 3, V). It is also destitute of radiating cracks or fragments such as would be likely to accompany the bursting of the wall by pressure. By pressing the cover slip with a needle so as to produce vertical and lateral movements the angle between a germ tube and the wall through which it has passed may be varied more than 90°. This is caused by the pressure of the liquid against the filament, which changes its position in the wall without bending it at the edge of the hole. The two ends of such a filament usually move in opposite directions, especially when the part extending through the wall is three or four times as long as the part in the original cell. If the filament were rigidly fixed in the wall, the angle between it and the wall would not change unless the filament were bent at the edge of the hole. Moreover, an occasional filament can be made to slide in the hole. These phenomena are possible only when the hole is larger than the filament. In view of these facts, it would seem that this fungus invades the cells by means of a cellulose-dissolving enzym (cellulase) secreted by the growing tips while in contact with the wall.

COMPARISON WITH BACILLUS CAROTOVORUS

As this Oospora and *Bacillus carotovorus* [3] cause similar rots of tomato fruits, a comparison of their effects on the host is interesting. Oospora invades the cells and destroys the protoplasm before it causes much separation of the walls (Pl. 1, B–F). Although it is also found in the intercellular spaces, it apparently makes little use of them except as passages. *Bacillus carotovorus*, on the other hand, remains in the intercellular spaces until it destroys the middle lamellae of the adjoining cell walls (Pl. 2, A–D) and enters the cells usually after it has destroyed their coherence. That an earlier entrance is sometimes effected, however, is evident from Plate 2, E. In later stages (Pl. 2, F–H) it not infrequently fills the cells.

ENZYM VERSUS PRESSURE

If we assume that the penetration of the cuticle by all fungous parasites is by "sheer mechanical presssure," as concluded for *Botrytis cinerea* by Brown (*4*, *5*) and by Blackman and Welsford (*1*) and for *Sclerotinia*

[3] An excellent history of the work done on the soft rots of vegetables (also tomato) caused by the *Bacillus carotovorus* group of organisms is published in "An Introduction to Bacterial Diseases of Plants," by Erwin F. Smith (*9*).

Libertiana by Boyle (*2*), we may be greatly misled. This would apply also to the conclusions of Hawkins and Harvey (*7*) regarding the penetration of cellulose walls. Fungi produce a variety of enzyms by means of which they decompose substances and obtain food. Moreover, it is quite likely that they produce many more enzyms than have been isolated. Failure to isolate an enzym therefore does not disprove its existence, especially as the physiological factors involved in the production of enzyms and the activators and inhibitors controlling their activities are little understood. Moreover, the observation by Hasselbring (*6*) that the cavities made in the waxy covering of *Berberis Thunbergii* berries by the anthracnose fungi are much larger than the germ tubes and the conclusions by Ward (*10*, *11*) from an exhaustive study of the brownrust of bromes that there is absolutely no relation between thickness of walls, number and size of stomates, hairs, and other mechanical structures and immunity to rust, present a striking contrast to the pressure theory of parasitic invasion. That growth pressure often accompanies enzymic action is obvious. It may also speed up the process of penetration and in certain cases serve as the chief, if not the sole, means of invasion, but it plays only a minor and nonessential part in the invasion of the tomato fruit cells by *Oospora lactis* and no doubt acts only in a secondary capacity in most cases. This is fortunate, for if the development of disease-resistant fruits depended upon thickness of walls, quality and resistance would often be diametrically opposed.

Since Oospora passes through the cellulose walls of two adjoining fruit cells without attaching itself to the wall or protoplasm and not infrequently makes an opening larger than the filament without causing a depression in the wall of the occupied cell, nor lateral cracks, or ruptures, in the wall of the invaded cell, its means of penetration can not be ascribed to pressure. The only other known means by which a fungus can make an opening of this description is by the use of an enzym, such as cellulase, which has been isolated from fungi.

There are features in the penetration of cell walls by some fungi, especially species of the genus Pythium, which cast considerable doubt on the penetration of cell walls by pressure. In his study of *Pythium gracile*, Ward observed that an oospore which had germinated at some little distance from a cress seedling, produced as it grew several bends in its germ tube and passed around a small algal cell at right angles before reaching a cress cell. On coming into contact with this cress cell its "apex became closely pressed against the cuticle, apparently lifting the whole hypha slightly in the process," evidently a result of pressure, but thereafter made no further movement nor change in its position as it produced a small hole in the cuticle and cellulose wall, passed through, and enlarged to normal size within the cell. As this fungus filament consisted of a single cell, the pressure within it was equally distributed. If the pressure had been sufficient to penetrate the host cell wall, which was many times as thick as the fungus wall, it would have straightened the fungus filament. As a matter of fact, however, it did not straighten a single bend nor change its position in the least, although such filaments bend easily, even by the motion of delicate water currents. Moreover, if pressure had caused the penetration of the wall, the fungus would have made a hole as large as its filament instead of a small hole, for the pressure on every unit area of its wall surface was equal. A more probable cause of this type of penetration is that cellulase is formed solely at a single point on the tip of the filament.

In view of the foregoing evidence, it would seem that the pressure theory of cell wall penetration by fungi is not so well supported as the enzym theory.

DISTRIBUTION OF THE FUNGUS

As this physiological form of *Oospora lactis* has been isolated frequently from tomato fruits shipped from the Gulf States, it is probably common in at least several of the Southern States. It is difficult from the reports of the Bureau of Markets' inspectors to estimate how often it really occurs in shipped tomatoes, as they report all such rots as "soft rot." It was found to be common at the Government Experimental Farm, Arlington, Va., and in gardens in the vicinity of Washington, D. C., where no doubt it has carried on its parasitic activities for some time, but has been overlooked because of the similarity of the rots produced by it and *Bacillus carotovorus* and the not infrequent association of the two organisms in the same fruits.

POSSIBLE MEANS OF CONTROL

As this fungus infects tomato fruits quite readily between 9° and 32° C. and can infect them at temperatures ranging from 2° to 37.5°, the practicability of controlling it by means of low temperatures seems doubtful.

Some experiments were made to determine the effects of antiseptics on the control of this organism. The fungus was immersed for 30 minutes in an aqueous solution of the antiseptic and transferred to sterile carrot decoction, in which, if it were still viable, it grew readily. The results are summarized in Table III.

TABLE III.—*Effect of antiseptics on the viability of Oospora lactis parasitica*

Antiseptic.	Concentration of solution.		Subsequent growth in carrot decoction.
	Antiseptic.	Water.	
Chlorid of lime	1	40	None.
Potash alum	(a)	(a)	Considerable.
Potassium permanganate	1	400	None.
Copper sulphate	(a)	(a)	Good.
Mercuric chlorid	1	5,000	None.
Formaldehyde, 37 per cent	1	300	Do.
Water			Abundant.

a Saturated.

Immersing the fungus for 30 minutes in an aqueous solution of chlorid of lime 1:40, potassium permanganate 1:400, formaldehyde (37 per cent) 1:300, or mercuric chlorid 1:5000, prevented its subsequent growth, but a similar treatment with a saturated solution of potash alum or copper sulphate was ineffective. The resistance of this fungus to copper sulphate—a fungicide of wide use—is surprising. Although the experiments with this treatment were repeated several times, only negative action was obtained.

Some experiments were also made on the use of the antiseptics as washes. Tomato fruits varying in maturity from green to ripe were lightly pricked in several places and submerged for 5 minutes in an aqueous suspension of Oospora spores, then drained, washed in an antiseptic solution for 30 minutes, and placed in moist chambers. From 20

to 40 pricked fruits, 8 unpricked fruits, and a number of controls equal to the number of treated fruits were used in each treatment. The results are summarized in Table IV.

TABLE IV.—*Effect of washing green to ripe tomato fruits for 30 minutes in an antiseptic to control watery-rot*

Fungicide.	Strength of solution; parts antiseptic to water.	Pricked fruit infected 6 days after treatment.	Unpricked fruit infected 12 days after treatment.
		Per cent.	*Per cent.*
Potash alum	1:50	31	25
Do	1:40	30	.....
Chlorid of lime	1:50	20	.....
Do	1:40	14	12½
Formaldehyde (37 per cent)	1:300	30	0
Do	1:240	22	0
Control	.....	57	37½

The percentage of infected fruits is higher in the pricked than in the unpricked series. Moreover, it is higher than in shipped fruits. As shipped fruits usually become infected through the stem scar instead of through the epidermis, they are probably comparatively free from punctures and therefore compare more nearly with the unpricked series. Formaldehyde and chlorid of lime (calcium hypochlorite) caused considerable reduction in the percentage of infected fruits. It is quite likely that sodium hypochlorite, which is a cheap convenient solution, would give more effective results than chlorid of lime. As the latter is partly insoluble it should, if used on a commercial scale, be dissolved in a separate tank so the clear liquid can be drawn off for use, or dissolved in a washing tank having a removable perforated sheet a few inches above the bottom to prevent the solid particles from adhering to the fruits.

The lower effectiveness of these antiseptics in the presence of fruits as compared with their effect on free spores (Table III) is probably due to chemical reaction of the germicides with substances on the surface of the fruits, which reduces their strength.

The economical use of a fungicide for washing tomato fruits should not be measured by its effect on a single rot, for if it controls one, it will give at least partial control of several others that cause heavy loss during shipment. The selection of a solution for this purpose should therefore be based on its control of this whole group of rots as well as on its cheapness and ease of handling.

SUMMARY

(1) A new rot of tomato fruits, closely resembling the rot caused by *Bacillus carotovorus*, has been common since the spring of 1921 in tomatoes shipped from the Gulf States. It is also prevalent in the vicinity of Arlington, Va., and Washington, D. C.

(2) The rot is characterized by dark-colored water-soaked areas which start in the stem scar and spread slowly toward the blossom end of the fruit without the production of a pronounced odor.

(3) The causal organism is a physiological form of *Oospora lactis*, which, though incapable of penetrating the epidermis, usually enters the fruits through the stem scar, but may enter through any place not cov-

ered by the cuticle, such as wounds, cracks, and punctures. It shows a little preference for ripe fruits, but infects green fruits quite readily. It invades the cells, destroys their protoplasmic contents, and causes loss of cell coherence through a slow dissolution of the middle lamellae of the cell walls.

(4) The minimum temperature for the germination of its spores, growth of its mycelium, and its infection of tomato fruits is approximately 2° C., the optimum 30°, and the maximum between 37.5° and 38.5°.

Immersing the fungus spores (cells) in an aqueous solution of an antiseptic for 30 minutes and transferring them to sterile carrot decoction had the following effects on their viability: Chlorid of lime 1:40, potassium permanganate 1:400, formaldehyde (37 per cent) 1:300, and mercuric chlorid 1:5,000, no growth; potash alum or copper sulphate in saturated solution, considerable growth. The percentage of tomato fruits infected after inoculation with this fungus was considerably reduced by washing them for 30 minutes in an aqueous solution of chlorid of lime 1:40 or formaldehyde (37 per cent) 1:240. It would seem from these results that an effective wash could be developed for the control of a large number of these rots during shipment.

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PLATE 1

Sections of a 2-day-old spot of a green tomato fruit affected by watery-rot (*Oospora lactis parasitica*.

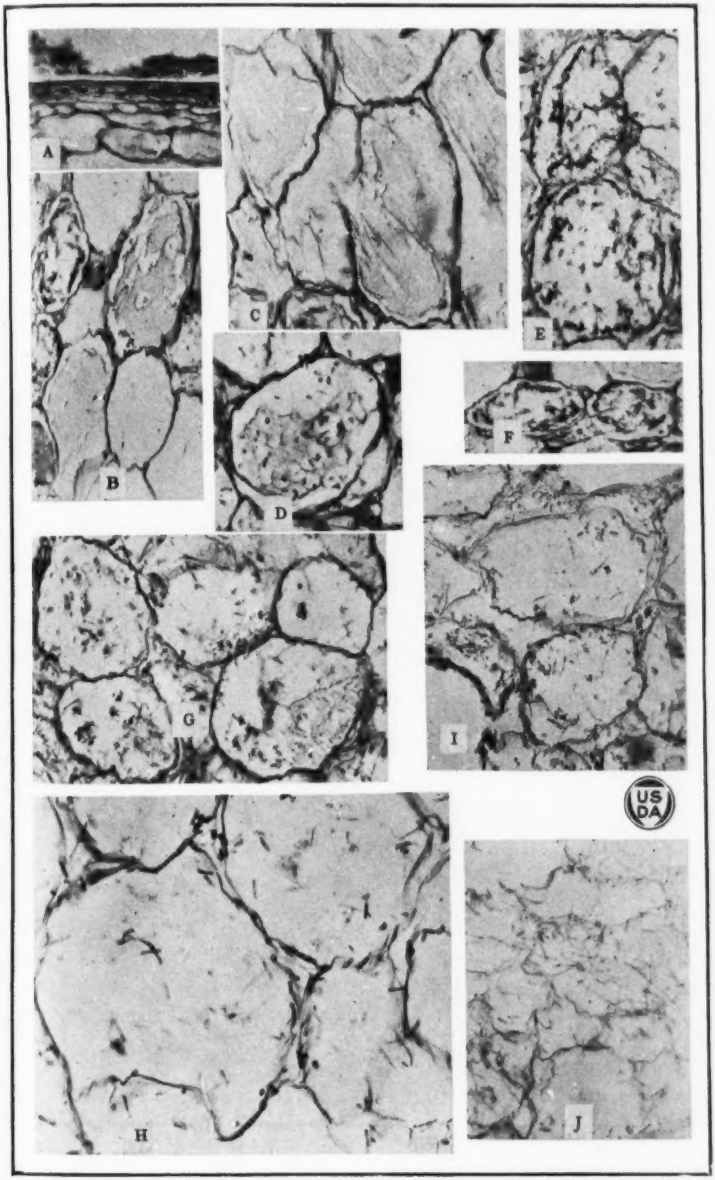
A.—Masses of the fungus lying on the surface unable to penetrate the cuticle.

B–G.—Consumption of the protoplasm by the fungus; B–C, early stages; D, medium early stage; E–G, late stages.

H.—Cells beginning to lose their coherence after destruction of their protoplasm.

I.—A more complete stage of cell separation than shown in H.

J.—Very late stage. Protoplasmic contents completely destroyed; cells free; walls thin, irregular, and inconspicuous.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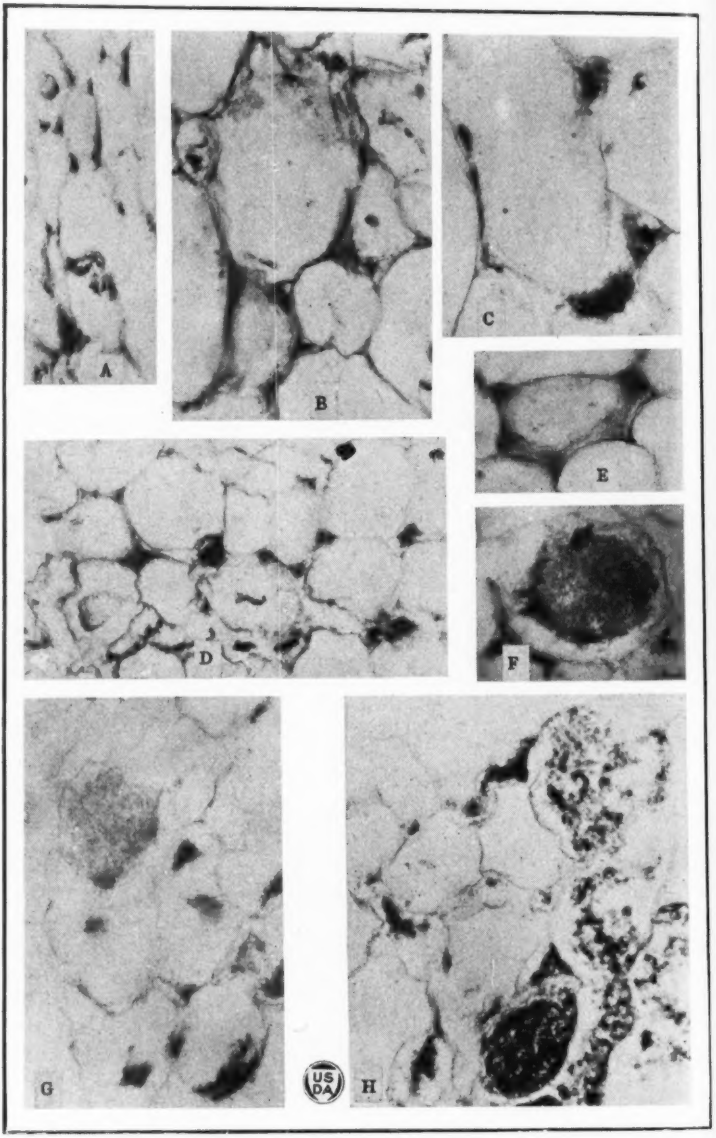
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PLATE 2

Sections of a tomato fruit infected by *Bacillus carotovorus*.

A.—Early stage of invasion; bacteria confined to the intercellular spaces.

B–D.—Medium early stages. B–C.—Intercellular spaces enlarging through dissolution of the middle lamellae of the adjoining cell walls; bacteria beginning to invade the cells.

D.—Cells losing their turgidity and coherence; middle lamellae dissolved.

E.—Bacterial invasion of a firmly attached cell.

F–H.—Late stages. Cells occupied by the bacteria.

PLATE 3

Oospora lactis parasitica

A–D.—Types of branches. A.—Short branches which arise near the septa at an angle of about 45° with the filament and break up into more or less rounded, irregularly shaped cells which function as spores. B–D.—Long branches which bear lateral branches of type A, but also break up into numerous cells capable of immediate germination.

E–F.—Segmented branches of type A; also early stages in the segmentation of the central filament.

G.—Order of segmentation in branch of type A.

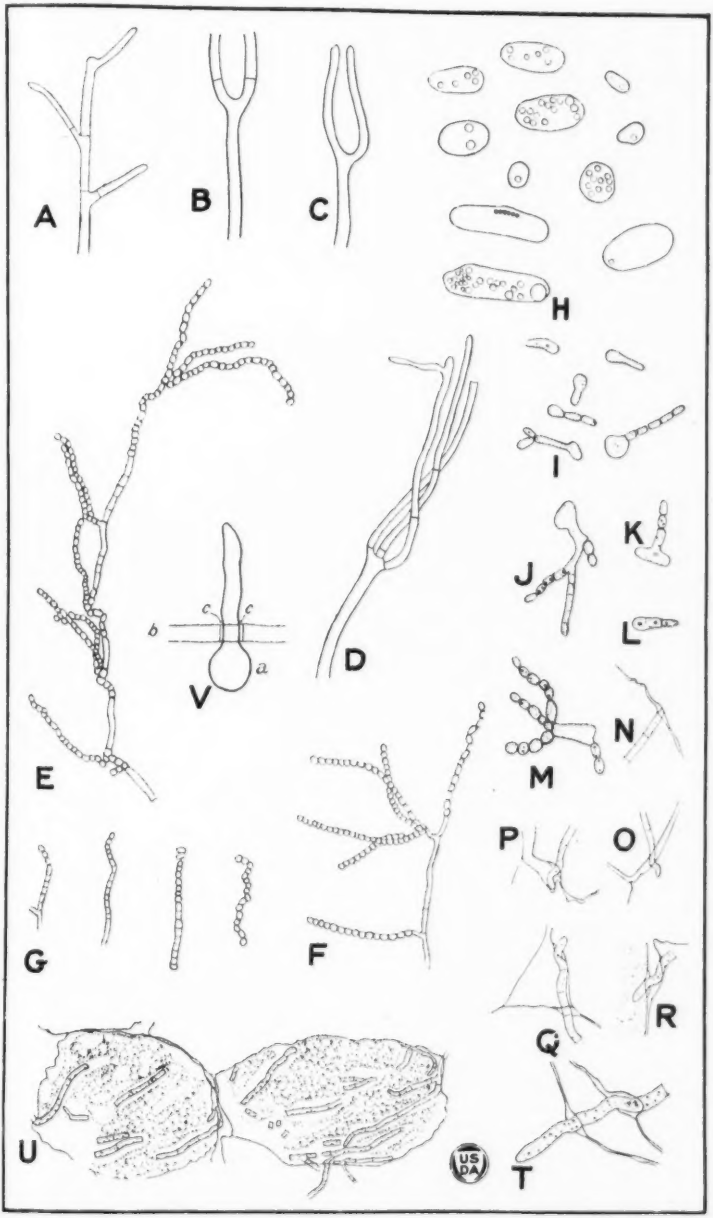
H.—Highly magnified detached cells of lateral branches and central filament.

I–M.—Germinating detached cells.

N–T.—Penetration of irregular, wavy, somewhat separated walls of partly destroyed cells by tips of growing hyphae.

U.—Cells of tomato fruit tissue containing hyphae surrounded by digestion vacuoles.

V.—Penetration of the cell wall of a tomato fruit cell by a germinating spore. (*a*) Germinating spore. (*b*) Cell wall. (*c*) Hole made in the wall by the germ tube. Somewhat diagrammatic.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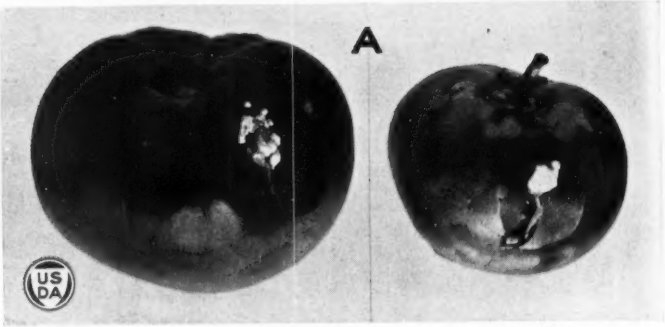
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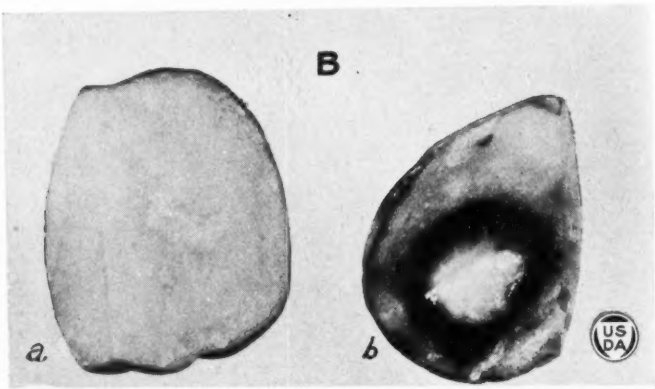
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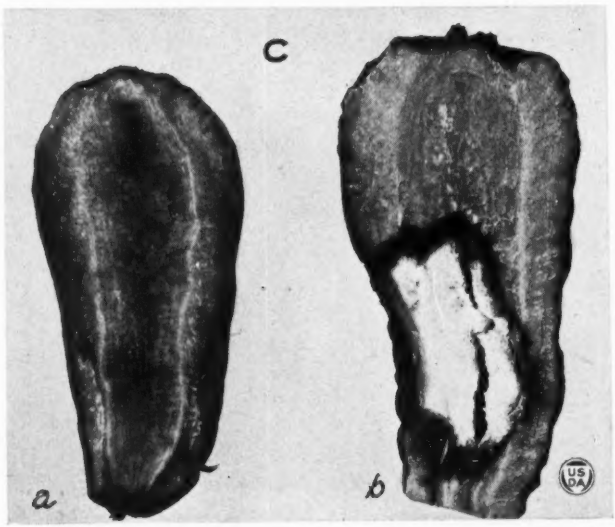
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PLATE 4

A.—Watery-rot of tomato fruits produced by *Oospora lactis parasitica*. The fruits were inoculated in needle punctures below the stem scar and kept in a moist atmosphere.

B.—Halved potato tubers. (*a*) Inoculated with *Oospora lactis parasitica;* (*b*) inoculated with *Bacillus carotovorus*.

C.—Halved carrot roots. (*a*) Pricked with a sterile needle; (*b*) inoculated with *Oospora lactis parasitica* isolated from watery-rot lesions of tomato fruit.

INFLUENCE OF THE ABSOLUTE REACTION OF A SOIL UPON ITS AZOTOBACTER FLORA AND NITROGEN FIXING ABILITY[1]

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INTRODUCTION

In a preliminary report the writer (*2*)[2] called attention to the apparent close correlation existing between the absolute reaction of a soil extract and the presence of Azotobacter in the soil. The data presented in this report showed that when the hydrogen-ion concentration of the soil extract exceeded 1×10^{-6} the soil, with very few exceptions, failed to initiate the growth of Azotobacter when introduced into a suitable mannite culture solution. On the other hand, when the hydrogen-ion concentration of the soil extract was less than 1×10^{-6} similar cultures almost always developed typical Azotobacter films. The data, though meager, indicated that the maximum hydrogen-ion concentration endured by Azotobacter in soils was near that represented by a P_H of 6.

In the preliminary report only 90 soils were examined and the hydrogen-ion concentration of the soil extract was determined by the Clark and Lubs colorimetric method as modified for soils by Gillespie (*7*). Some investigators place little credence in the colorimetric method for determining hydrogen-ion concentrations, particularly in a medium as complex as a soil extract. Gillespie, however, found only slight differences in the hydrogen-ion concentration of soil extracts determined by this method and in suspensions of the same soils determined electrometrically. Since the publication of the preliminary report 418 soils from widely varying localities and conditions have been subjected to similar examinations, the resulting data being the basis of this paper. The hydrogen-ion concentration of these soils has been determined colorimetrically upon an extract and electrometrically upon a suspension of the soil.

The soils of Series I were collected within a few miles of the station either by the writer or one of his assistants. These soils represent practically all types and conditions of soil found in this immediate vicinity. Those of Series II were also collected near the station and in many instances at or near the point where soils of Series I were taken. These collections were made 2 years later to serve as a control on the first examinations. Where soils are duplicates of former samples, it has been indicated in Table VII by recording the former numbers. The soils of Series III were collected, as indicated in Table IX, from different counties in the State of Kansas by the county agents. Sterile containers were sent out and requests were made to collect soils representative of conditions in the area covered. The directions called for removing the inch or two of surface soil and collecting from at least four points within a short distance of each other in order to obtain as far as possible a representative sample of the immediate area. In Series IV the soils

[1] Accepted for publication Jan. 22, 1923. Contribution No. 51, Department of Bacteriology, Kansas Agricultural Experiment Station.

[2] Reference is made by number (italic) to "Literature cited," p. 937–938.

were obtained from a number of different States. These were secured, in most instances, through the various experiment stations. The collector was requested to send samples, where possible, from alkaline or limed, and acid or unlimed, adjacent soils.

METHODS

Soon after the soils reached the laboratory they were well mixed and four 300 cc. Erlenmeyer flasks containing 50 cc. of mannite cultural solution were inoculated from each soil. Ten cubic centimeters of a suspension prepared by shaking 1 part of soil with 2 parts of sterile water was used as an inoculum. The suspension was allowed to stand a few minutes to let the heavier soil particles settle out. Two of the cultures were immediately sterilized in the autoclave to act as controls on total nitrogen determinations.

The culture medium employed had the following composition:

Magnesium sulphate	0.2 gm.
Di-basic potassium phosphate	0.2 gm.
Sodium chlorid	0.5 gm.
Ferric chlorid	Trace.
Calcium chlorid	Trace.
Mannite	20.0 gm.
Distilled water	1,000.0 cc.

This medium was rendered slightly alkaline to phenolphthalein with sodium hydroxid. In all experiments, except those reported in Table V, a small quantity of sterile calcium carbonate was added to each culture flask before inoculating. In all cases the cultures were incubated at room temperature for 3 weeks, after which total nitrogen determinations were made according to the modification of the Kjeldahl method suggested by Latshaw (*10*). The quantities of nitrogen reported represent the average of duplicate cultures after deducting the average of duplicate controls.

During the incubation period frequent examinations were made both macroscopically and microscopically, to ascertain the character of the growth. When "no film" is reported, no growth resembling Azotobacter took place during the first two weeks of incubation. After approximately 2 weeks of incubation a heavy fungus growth usually appeared, especially where no Azotobacter growth, or a nontypical Azotobacter film developed. After the development of a fungus film the growth became so complex that it was difficult to detect Azotobacter either macroscopically or microscopically. Such was the appearance of those cultures in which Azotobacter is reported as questionable. It is believed that the results would have been more consistent and striking if incubation had been reduced to 2 weeks. This would have avoided, to a large extent, the complications arising from the growth of fungi.

The microscopic examinations were made by placing on a slide a loop of that part of the surface growth which appeared most characteristic of Azotobacter, covering with cover glass, and examining with the 1/6 objective. If typical Azotobacter were present in appreciable numbers, the picture was so striking as to be almost unmistakable. If Azotobacter are not present in a soil in sufficient numbers and vigor to develop a visible film or to produce sufficient growth to be observed microscopically by the methods employed, it is questionable whether they are of any significance in the nitrogen economy of a soil.

In this examination of soils for Azotobacter it will be noted that three methods of detecting their presence were employed—the formation of a film, the microscopic examination, and the quantity of nitrogen fixed. Under the heading "Azotobacter" in Tables V, VII, IX, and XI a + (plus) sign has been placed where, in the opinion of the writer, the major evidence indicated the existence of a vigorous Azotobacter flora in the original sample of soil and a − (minus) sign where the evidence did not indicate the presence of a vigorous Azotobacter flora.

The colorimetric hydrogen-ion determinations were made by the Clark and Lubs method as modified for soils by Gillespie. The soils were ground to pass a 40-mesh sieve and a weighed quantity mixed with five times its weight of water, shaken well and centrifugalized until the supernatant liquid was practically clear. The water used in the preparation of the soil extract and suspension was freshly distilled from a mixture of sulphuric acid and potassium dichromate into a flask containing barium hydroxid. From this it was distilled into a third empty flask and again distilled. The P_H of water thus obtained was from 5.7 to 6.0 and was affected by the minutest trace of acid or alkali. All glassware coming in contact with the soil extract was washed in this water. Buffer solutions were prepared according to Clark and Lubs (*1*) and were checked, and adjusted if necessary, at frequent intervals on a Leeds and Northrup type K potentiometer.

Electrometric hydrogen-ion concentrations, or differences in potential between the soil solution and the hydrogen electrode, were made by using a Leeds and Northrup type K potentiometer in connection with saturated KCl − calomel and hydrogen electrodes similar to the one described by Hildebrand (*9*). The ratio of soil to water used was the same as employed in colorimetric determinations; i. e., 1 to 5. Six hydrogen electrodes were connected by switches to the potentiometer so that six samples could be run at the same time. Hydrogen was bubbled through the cells continuously at a rather rapid rate, the cells being constantly shaken. A maximum difference in potential was usually recorded in 10 to 30 minutes, after which the difference decreased very slowly. The length of time required to reach the maximum reading apparently depended, other things being equal, upon the rate of flow of hydrogen. The influence of the rate of flow of hydrogen upon the length of time necessary to obtain maximum difference of potential is illustrated in the data presented in Tables I and II. Neutral or alkaline soils usually required a longer time to reach the maximum difference in potential, and the agreement between duplicates was not, as a rule, as close as it was with acid soils.

TABLE I.—*Time required for electrodes to record maximum difference in potential; hydrogen passed over electrodes slowly (readings recorded as millivolts)*

Soil No.	Time (in minutes).													
	15	20	30	35	40	45	50	55	60	70	80	90	95	100
376	613	....	625	....	....	....	655	....	673	683	687	690	....	[a]693
376	618	....	654	....	....	....	678	....	685	690	694	695	....	[a]696
380	503	....	515	....	....	554	563	565	[a]566	....	....	....	....	....
380	517	....	540	....	....	563	[a]566	566	566	....	....	....	....	....
381	....	520	....	523	....	[a]524	....	524	....	....	....	....	....	....
381	....	500	....	524	....	[a]526	....	526	....	....	....	....	....	....
410	....	633	....	....	642	....	....	670	....	695	....	....	[a]703	703
410	....	654	....	....	691	....	....	697	....	[a]702	....	....	702	700

[a] Maximum reading.

TABLE II.—*Time required for electrodes to record maximum difference in potential; hydrogen passed over electrodes rapidly (readings recorded as millivolts)*

Soil No.	Time (in minutes).						
	5	10	15	20	25	30	35
74	678	688	[a] 691	690	690	.....	.....
74	668	681	680	[a] 682	682	.....	.....
75	674	683	[a] 688	688	688	.....	.....
75	670	685	688	690	[a] 692	.....	.....
76	583	[a] 587	586	585	584	.....	.....
76	590	[a] 592	591	590	588	.....	.....
24	648	.....	[a] 675	.....	674	.....	.....
24	630	.....	[a] 667	.....	664	.....	.....
26	662	.....	[a] 670	.....	666	.....	.....
26	665	.....	[a] 670	.....	670	.....	.....
28	[a] 690	.....	690	.....	686	.....	.....
28	682	.....	680	.....	[a] 688	.....	.....
209	[a] 576	574	573	.....	.....	.....	.....
209	535	570	[a] 577	.....	.....	.....	.....
210	540	[a] 546	546	.....	.....	.....	.....
210	536	546	[a] 550	.....	.....	.....	.....
191	.....	.....	[a] 562	.....	562	.....	560
191	.....	.....	564	.....	[a] 566	.....	564
192	.....	.....	[a] 562	.....	560	.....	558
192	.....	.....	560	.....	[a] 564	.....	564
265	.....	.....	[a] 515	.....	515	514	.....
265	.....	.....	[a] 515	.....	515	514	.....
166	575	.....	.....	[a] 588	.....	586	.....
166	565	.....	.....	[a] 586	.....	584	.....
38	556	.....	.....	[a] 563	.....	562	562
38	525	.....	.....	557	.....	[a] 561	560
8	548	.....	.....	582	.....	[a] 585	585
8	548	.....	.....	577	.....	584	[a] 585

[a] Maximum reading.

The platinum electrodes used were coated with platinum black and tested on a standard acetate solution before using. Several determinations could usually be run with one coating of platinum black. Duplicate samples of soil were always run and as a rule the results agreed within 10 millivolts. If the disagreement were much greater than this, the sample was again run. Sharp and Hoagland (*13*) state that "Duplicate determinations on soil suspension usually agreed within 0.01 to 0.02 volt." Plummer (*11*) says "Duplicate readings on the same sample of soil could easily be read to 0.02 volt," while "It was almost impossible to get such closely agreeing results as 0.02 volt with different samples of the same soil." The data presented in Tables I, II, III, and IV illustrate the millivolt readings of duplicate samples run on different electrodes. In Table IV are shown the millivolt readings of samples of the same soil run on different dates and also the slight effect upon the reading of varying the ratio of soil to water.

TABLE III.—*Effect on electrode readings of varying the method of saturating the electrode and suspension with hydrogen*

Soil No.	Hydrogen run over electrode continuously.					
	Electrode number—					
	1	2	3	4	5	Average.
401	615	608	595	601	619	608
362	557	550	552	553	554	553
378	542	544	541	541	538	541
377	545	542	549	541	551	546

Soil No.	1,000 cc. hydrogen run over electrode.					
	Electrode number—					
	1	2	3	4	5	Average.
401	[a] 597	[a] 611	[a] 611	[a] 615	611	609
362	550	550	550	550	.....	550
378	543	542	530	540	538	539
377	550	[a] 530	[a] 537	[a] 538	554	542

Soil No.	1,000 cc. hydrogen run over electrode, then run continuously.					
	Electrode number—					
	1	2	3	4	5	Average.
401	629	620	622	624	614	622
362	553	553	557	555	.....	554
378	543	543	533	543	530	538
377	555	554	550	552	552	553

[a] These samples apparently did not have sufficient hydrogen passed through to saturate the electrode and suspension, as is evidenced by the increased reading when further passage of hydrogen took place.

TABLE IV.—*Difference in potential of same soil determined on different dates, effect of varying ratio of soil to water, and variations in difference in potential as determined with five different electrodes*

Soil No.	Ratio of soil to water	Electrode number—					
		1	2	3	4	5	Average
390 [a]	1 to 5	534	544	541	539	532	537
390 [b]	1 to 5	538	540	539	532	536	537
369	1 to 5	678	679	677	678	676	678
395	1 to 5	530	528	528	528	529	529
395	1 to 10	536	533	536	536	539	536
387	1 to 5	493	494	493	493	493	493
387	1 to 10	495	494	494	495	494	494
384	1 to 5	650	653	650	652	652	651
397	1 to 5	563	566	567	566	566	566

[a] April 5. [b] May 31.

In converting volt readings into P_H use has been made of the tables prepared by Schmidt and Hoagland (*12*) adding 91 millivolts to the readings to convert them into N/10 KCl–calomel electrode readings. Some investigators regard the difference in potential between saturated and N/10 KCl–calomel electrodes to be of a value other than 91 millivolts. To convert the P_H values here recorded into those of any other difference in potential between saturated and N/10 KCl–calomel electrodes, it is only necessary to add or subtract, as the case may be, 0.017 from the figure here recorded for each millivolt above or below 91. All determinations were made at room temperature, and Schmidt and Hoagland temperature correction factors were used to convert room temperature readings into 25° C. readings.

Hydrogen was purchased in cylinders and washed through a saturated solution of mercuric chlorid, alkaline potassium permanganate solution, alkaline pyrogallic acid solution, and distilled water before entering the hydrogen electrode cell. The connection between the calomel and hydrogen electrodes was made through a glass stopcock. The end of this immersed in the soil suspension was drawn out to a capillary opening and the cock was kept closed during the determination. Between successive determinations, however, the connection was refilled with fresh saturated potassium chlorid.

RESULTS

Data relative to the type of growth and the quantity of nitrogen fixed in culture media inoculated from soils, together with the absolute reaction of the soil extract determined colorimetrically and the soil suspension determined electrometrically of 418 soils, are recorded in Tables V, VII, IX, and XI. In Tables VI, VIII, X, and XII some of the data have been rearranged to show the possible association, or correlation, existing between the hydrogen-ion concentration of the soil solution and the presence or absence of Azotobacter in the soils. In these various tables under the heading "Azotobacter" a + (plus) sign has been recorded to indicate the presence of Azotobacter and a − (minus) sign to indicate the absence of Azotobacter in the soils. These data are not absolute. Sometimes it was very difficult to differentiate between the presence or absence of Azotobacter, There are a few instances where an Azotobacter film developed in only one of duplicate samples, or where either the film or Azotobacter cells were questionable, accompanied by poor fixation of nitrogen, that Azotobacter have been recorded as absent. On the other hand, there are a few instances where similar conditions were accompanied by good fixation of nitrogen that have been recorded as containing Azotobacter. An effort has been made to weigh the evidence in questionable cases as carefully as possible and record Azotobacter as present if it were believed that the data indicated the presence of an active Azotobacter flora, and as absent if the available data did not indicate the presence of such a flora.

It should be borne in mind that the chance for contamination of Azotobacter free soils with Azotobacter was not entirely eliminated. Especially was this true in Series III and IV, for nearly all of the soils in these two series were collected by men inexperienced in bacteriological technic and were shipped long distances. Sometimes one or more of the individual containers were broken open upon arrival at the laboratory.

Even in the laboratory the possibility of contamination could not be entirely eliminated. What influence such contamination may have had upon the development of Azotobacter from soils that normally contained no Azotobacter is not known.

Again, it has been shown (*3*) that Azotobacter can exist for varying lengths of time in soils that will not support them indefinitely. Under natural conditions, if Azotobacter exists in the proximity of soils not containing them, almost constant inoculation due to wind, rain, animals, cultivation, etc., is inevitable. The length of time that such introduced organisms may remain in a viable condition apparently depends upon the intensity of the unfavorable influences. It is, therefore, highly probable that Azotobacter may frequently be isolated from soils in which they will not function or even exist for a very long period.

On the other hand, little is known as to how many Azotobacter are necessary to initiate the development of a visible film in laboratory culture media. Unpublished data indicate that appreciable numbers are essential to the development of a characteristic film. Also, nothing is known as to how rapidly they may disappear from a sample of soil removed from its natural environment. It is possible, therefore, that Azotobacter may be present in relatively large numbers in a soil and escape observation by the methods employed in these investigations.

In comparing the reaction with the presence or absence of Azotobacter, and in obtaining a mathematical expression for this association or correlation (the association coefficient), the soils have been divided into two groups; those with a P_H of 6.0 or above and those more acid than P_H 6.0. This division point has been chosen more or less arbitrarily, though the data here presented indicate that it is not far from absolute. The association coefficient was obtained by the use of Yule's (*14*) association coefficient formula, as mentioned elsewhere (*5*). Association coefficients have been calculated both from the data secured by the colorimetric and the electrometric P_H determination. In analyzing the data presented here it will perhaps be best to examine those from each series of soils separately.

SERIES I

The data secured from an examination of the soils of Series I are presented in Table V, rearranged in part in Table VI, and summarized below. These soils together with those of Series II are all local soils and were, perhaps, collected and handled with more care than those of Series III and IV. Samples 1 to 19 were lost through error before the electrometric hydrogen-ion determinations were run. However, in the following summary the electrometric P_H of samples No. 1, 4, 5, 14, and 15 have been regarded as above P_H 6.0 and all other soils below No. 20 as more acid than P_H 6.0. These 19 soils could not be considered in obtaining the numerical average of colorimetric P_H. Likewise samples 85-90, for which no quantitative nitrogen determinations were made, were omitted from the calculations for average nitrogen fixed.

TABLE V.—*Type of growth, nitrogen fixed, and reaction of soils of Series I*

Soil No.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
			Mgm.			
1	Typical Azotobacter	Typical Azotobacter	10.3	+	6.9	.....
2	None	No Azotobacter	2.0	−	5.4	.....
3	do	do	3.0	−	5.6	.....
4	Typical Azotobacter	Typical Azotobacter	6.8	+	6.9	.....
5	do	do	5.4	+	7.1	.....
6	None	No Azotobacter	4.4	−	5.7	.....
7	do	do	4.4	−	5.7	.....
8	do	do	4.6	−	5.6	.....
9	do	do	4.7	−	5.5	.....
10	do	do	−0.6	−	5.6	.....
11	do	do	1.7	−	5.8	.....
12	do	do	4.5	−	5.9	.....
13	do	do	3.2	−	5.6	.....
14	Typical Azotobacter	Typical Azotobacter	8.5	+	7.4	.....
15	do	do	10.3	+	7.4	.....
16	None	No Azotobacter	4.0	−	5.6	.....
17	do	do	3.0	−	5.5	.....
18	do	do	3.7	−	5.6	.....
19	do	do	4.5	−	5.7	.....
20	do	do	4.7	−	5.4	5.10
21	do	do	4.7	−	5.6	5.05
22	do	do	4.7	−	5.6	5.27
23	do	do	4.7	−	5.6	5.10
24	do	do	4.2	−	5.6	4.90
25	Typical Azotobacter	Typical Azotobacter	6.4	+	7.0	6.45
26	do	do	9.9	+	6.6	6.13
27	do	do	7.5	+	6.1	5.88
28	do	do	9.5	+	6.2	5.86
29	do	do	8.8	+	7.6	7.27
30	None	No Azotobacter	4.1	−	5.6	5.39
31	Typical Azotobacter	Typical Azotobacter	10.6	+	7.5	7.71
32	None	No Azotobacter	5.6	−	5.9	5.85
33	do	do	4.0	−	6.7	6.56
34	do	do	4.5	−	6.8	6.74
35	Typical Azotobacter	Typical Azotobacter	6.8	+	7.6	7.62
36	do	do	9.2	+	6.0	6.12
37	None	No Azotobacter	5.4	−	6.2	6.07
38	Nontypical [a]	Azotobacter present [a]	3.9	−	5.6	5.59
39	do	do	8.6	+	6.1	6.05
40	do	do	8.1	+	7.0	6.72
41	do	Typical Azotobacter	7.6	+	6.0	5.98
42	do	do	8.3	+	7.4	7.05
43	Typical Azotobacter	do	9.6	+	7.7	8.75
44	Nontypical	do	9.9	+	7.5	7.71
45	Typical Azotobacter	do	10.1	+	7.4	7.45
46	None	No Azotobacter	1.0	−	5.9	6.03
47	Typical Azotobacter	Typical Azotobacter	10.0	+	7.4	7.52
48	Nontypical	do	6.6	+	6.4	6.52
49	None	No Azotobacter	2.7	−	5.5	5.03
50	do	do	4.0	−	5.8	5.32
51	do	do	3.2	−	5.3	5.41
52	Nontypical	Azotobacter present	5.5	+	7.3	7.27
53	Typical Azotobacter	Tppical Azotobacter	9.0	+	7.7	7.79
54	Nontypical	do	7.4	+	6.0	6.18
55	Typical Azotobacter	do	8.7	+	7.5	7.50
56	Nontypieal	do	7.2	+	7.4	7.18
57	Typical Azotobacter	do	7.6	+	7.5	7.30

[a] P_H not determined electrometrically.

TABLE V.—*Type of growth, nitrogen fixed, and reaction of soils of Series I*—Continued

Soil No.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
			Mgms.			
58	None	No Azotobacter	4.0	−	5.5	5.46
59	Typical Azotobacter	Typical Azotobacter	10.6	+	7.4	7.49
60	None	No Azotobacter	4.6	−	5.8	5.73
61	do	do	3.7	−	5.5	5.51
62	Typical Azotobacter	Typical Azotobacter	7.3	+	7.5	7.55
63	do	do	11.0	+	7.4	7.43
64	do	do	8.3	+	6.1	6.35
65	None	No Azotobacter	5.2	−	5.7	5.94
66	do	do	3.8	−	5.7	5.81
67	do	do	4.1	−	5.5	5.32
68	Nontypical	Typical Azotobacter	6.9	+	6.1	6.05
69	None	No Azotobacter	4.1	−	5.6	5.19
70	Typical Azotobacter	Typical Azotobacter	8.7	+	6.8	6.93
71	Nontypical	do	6.9	+	5.6	5.61
72	do	do	6.8	+	7.0	6.72
73	None	No Azotobacter	4.5	−	5.9	5.88
74	Nontypical [a]	Typical Azotobacter [a]	8.1	+	7.4	7.25
75	Typical Azotobacter	do	6.1	+	7.5	7.30
76	Nontypical [a]	do [a]	3.4	−	5.5	5.63
77	None	No Azotobacter	4.2	−	5.6	5.34
78	Nontypical	Typical Azotobacter	5.5	+	7.7	7.54
79	Typical Azotobacter	do	7.6	+	7.7	8.48
80	Nontypical	Azotobacter present	9.2	+	7.6	7.84
81	Typical Azotobacter	Typical Azotobacter	9.6	+	7.4	7.77
82	do	do	8.0	+	7.7	7.60
83	do	do	9.9	+	7.6	7.72
84	do	do	9.5	+	7.5	7.47
85	do	do	.....	+	7.5	7.62
86	do	do	.....	+	7.5	7.60
87	do	do	.....	+	7.8	7.59
88	do	do	.....	+	6.9	6.88
89	do	do	.....	+	7.5	7.76
90	do	do	.....	+	7.3	7.06

[a] P_H not determined electrometrically.

TABLE VI.—*Correlation between reaction and presence of Azotobacter in soils of Series I*

P_H determined electrometrically.						P_H determined colorimetrically.					
Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.
43	8.75	+	46	6.03	−	87	7.8	+	37	6.2	−
79	8.48	+	41	5.98	+	43	7.7	+	27	6.1	+
80	7.84	+	65	5.94	−	53	7.7	+	39	6.1	+
53	7.79	+	27	5.88	+	78	7.7	+	64	6.1	+
81	7.77	+	73	5.88	−	79	7.7	+	68	6.1	+
89	7.76	+	28	5.86	+	82	7.7	+	36	6.0	+
83	7.72	+	32	5.85	−	29	7.6	+	41	6.0	+
31	7.71	+	66	5.81	−	35	7.6	+	54	6.0	+
44	7.71	+	60	5.73	−	80	7.6	+	12[a]	5.9	−
35	7.62	+	76	5.63	−	83	7.6	+	32	5.9	−
85	7.62	+	71	5.61	+	31	7.5	+	46	5.9	−
86	7.60	+	38	5.59	−	44	7.5	+	73	5.9	−
82	7.60	+	61	5.51	−	55	7.5	+	11[a]	5.8	−
87	7.59	+	58	5.46	−	57	7.5	+	50	5.8	−
62	7.55	+	51	5.41	−	62	7.5	+	60	5.8	−
78	7.54	+	30	5.39	−	75	7.5	+	6[a]	5.7	−
47	7.52	+	77	5.34	−	84	7.5	+	7[a]	5.7	−
55	7.50	+	50	5.32	−	85	7.5	+	19[a]	5.7	−
59	7.49	+	67	5.32	−	86	7.5	+	65	5.7	−
84	7.47	+	22	5.27	−	89	7.5	+	66	5.7	−
45	7.45	+	69	5.19	−	14[a]	7.4	+	3[a]	5.6	−
63	7.43	+	20	5.10	−	15[a]	7.4	+	8[a]	5.6	−
57	7.30	+	23	5.10	−	42	7.4	+	10[a]	5.6	−
75	7.30	+	21	5.05	−	45	7.4	+	13[a]	5.6	−
29	7.27	+	49	5.03	−	47	7.4	+	16[a]	5.6	−
52	7.27	+	24	4.90	−	56	7.4	+	18[a]	5.6	−
74	7.25	+				59	7.4	+	21	5.6	−
56	7.18	+				63	7.4	+	22	5.6	−
90	7.06	+				74	7.4	+	23	5.6	−
42	7.05	+				81	7.4	+	24	5.6	−
70	6.93	+				57	7.3	+	30	5.6	−
88	6.88	+				90	7.3	+	38	5.6	−
34	6.72	−				5[a]	7.1	+	69	5.6	−
40	6.72	+				25	7.0	+	71	5.6	+
72	6.72	+				40	7.0	+	77	5.6	−
33	6.56	−				72	7.0	+	9[a]	5.5	−
48	6.52	+				1[a]	6.9	+	17[a]	5.5	−
25	6.45	+				4[a]	6.9	+	49	5.5	−
64	6.35	+				88	6.9	+	58	5.5	−
54	6.18	+				34	6.8	−	61	5.5	−
26	6.13	+				70	6.8	+	67	5.5	−
36	6.12	+				33	6.7	−	76	5.5	−
37	6.07	−				26	6.6	+	2[a]	5.4	−
39	6.05	+				48	6.4	+	20	5.4	−
68	6.05	+				28	6.2	+	51	5.3	−

[a] P_H not determined electrometrically.

The following summary of Tables V and VI needs little explanation.

Summary of Tables V and VI

Number of soils examined	90
Number of soils containing Azotobacter	51
Number of soils not containing Azotobacter	39
Average mgm. nitrogen fixed, 84 soils	6.25
Average mgm. nitrogen fixed, 45 soils containing Azotobacter	8.32
Average mgm. nitrogen fixed, 39 soils not containing Azotobacter	3.86
Number of soils electrometric P_H 6.0 or above	51
Number of soils electrometric P_H below 6.0	39
Number of soils colorimetric P_H 6.0 or above	53
Number of soils colorimetric P_H below 6.0	37
Number of soils electrometric P_H 6.0 or above containing Azotobacter	47
Number of soils electrometric P_H 6.0 or above not containing Azotobacter	4
Number of soils elcterometric P_H below 6.0 containing Azotobacter	4
Number of soils electrometric P_H below 6.0 not containing Azotobacter	35
Number of soils colorimetric P_H 6.0 or above containing Azotobacter	50
Number of soils colorimetric P_H 6.0 or above not containing Azotobacter	3
Number of soils colorimetric P_H below 6.0 containing Azotobacter	1
Number of soils colorimetric P_H below 6.0 not containing Azotobacter	36
Average electrometric P_H, 71 soils	6.57
Average colorimetric P_H, 90 soils	6.50
Average electrometric P_H, 46 soils containing Azotobacter	7.12
Average electrometric P_H, 25 soils not containing Azotobacter	5.57
Average colorimetric P_H, 51 soils containing Azotobacter	7.12
Average colorimetric P_H, 39 soils not containing Azotobacter	5.70
Association coefficient based on electrometric P_H determinations	0.981
Association coefficient based on colorimetric P_H determinations	0.997

It will be observed that the number of soils more alkaline than P_H 6.0 by either method and not containing Azotobacter and also the number more acid than P_H 6.0 containing Azotobacter are few. There were only four soils by the electrometric and three by the colorimetric method with a P_H of 6.0 or above that did not contain Azotobacter. One of the electrometric P_H 6.0 or above soils (No. 46) not containing Azotobacter was more acid than P_H 6.0 colorimetrically, while soil No. 37 was only slightly more alkaline than P_H 6.0 by both methods. There were four soils with an electrometric and one with a colorimetric reaction more acid than P_H 6.0 recorded as containing Azotobacter. Three of these gave a reaction less acid than P_H 6.0 by one of the methods and the remaining samples did not give a typical Azotobacter growth.

SERIES II

In Table VII are recorded the data collected from the examination of the soils of Series II. Many of these soils were collected near the point where certain soils of Series I were taken and may thus be regarded as controls on the earlier examinations. In Table XIII are recorded in parallel columns the data secured from the two examinations. It will be noted that the data are quite similar even though two years intervened between the two analyses. Part of the data of Table VII are rearranged in Table VIII to show the possible association existing between the presence of Azotobacter and the hydrogen-ion concentration of the soil solution.

TABLE VII.—*Type of growth, nitrogen fixed, and reaction of soils of Series II*

Soil No.	Duplicate soil.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
				Mgm.			
101	1	Typical Azotobacter.	Typical Azotobacter.	10.7	+	6.7	6.71
102	2	None.	No Azotobacter.	6.3	−	5.0	3.78
103	3	do.	do.	5.4	−	5.4	5.27
104	4	Typical Azotobacter.	Typical Azotobacter.	10.5	+	7.1	.....
105	5	do.	do.	11.8	+	7.2	7.08
106	6	None.	No Azotobacter.	6.1	−	5.7	5.25
107	31	Typical Azotobacter.	Typical Azotobacter.	10.2	+	7.4	7.70
108	32	None.	Azotobacter present.	8.5	+	5.6	5.48
109	33	Typical Azotobacter.	Typical Azotobacter.	9.5	+	6.6	6.47
110	34	do.	do.	7.9	+	7.4	7.61
111	35	do.	do.	9.1	+	6.6	6.39
112	.....	Nontypical Azotobacter.	Azotobacter present.	.....	+	5.8	5.48
113	36	Typical Azotobacter.	Typical Azotobacter.	8.9	+	5.6	5.85
114	37	Nontypical Azotobacter.	do.	8.9	+	5.8	5.59
115	38	Typical Azotobacter.	do.	8.6	+	5.4	5.51
116	39	do.	do.	10.0	+	6.0	5.90
117	.....	do.	do.	10.3	+	6.6	6.56
118	.....	do.	do.	8.9	+	7.5	7.28
119	40	do.	do.	9.8	+	7.1	7.00
120	61	Nontypical Azotobacter.	Azotobacter present.	6.0	+	5.8	5.68
121	66	None.	No Azotobacter.	7.6	−	5.7	5.24
122	64	Nontypical Azotobacter.[a]	Azotobacter present.	8.6	+	5.8	5.51
123	65	Typical Azotobacter.	Typical Azotobacter.	8.8	+	5.9	5.64
124	63	do.	do.	9.0	+	6.9	7.06
125	.....	None.	No Azotobacter.	4.7	−	5.5	5.15
126	49	do.	do.	8.0	−	5.5	5.19
127	50	do.	do.	6.6	−	5.5	5.10
128	51	do.	do.	5.1	−	5.9	5.78
129	52	Typical Azotobacter.	Typical Azotobacter.	10.8	+	7.6	7.30
130	53	do.	do.	7.3	+	7.4	7.52
131	54	None.	No Azotobacter.	7.7	−	5.8	5.47
132	.....	Nontypical Azotobacter.	Typical Azotobacter.	9.3	+	5.9	5.54
133	15	Typical Azotobacter.	do.	13.5	+	7.3	7.45
134	77	None.	No Azotobacter.	7.2	−	5.4	4.88
135	.....	Typical Azotobacter.	Typical Azotobacter.	11.7	+	7.2	7.20
136	16	None.	Azotobacter present.	10.5	+	5.4	4.88
137	17	do.	No Azotobacter.	4.3	−	5.8	5.58
138	18	do.	Azotobacter (?).	3.0	−	5.4	5.05
139	.....	do.	do (?).	8.9	+	6.2	5.90
140	.....	Typical Azotobacter.	Typical Azotobacter.	7.3	+	7.0	7.30
141	.....	do.	do.	7.9	+	7.2	7.48
142	82	do.	do.	9.7	+	7.8	7.59
143	79	do.	do.	7.0	+	8.1	8.77
144	80	do.	do.	8.8	+	7.3	7.72
145	83	do.	do.	7.5	+	7.2	7.94
146	.....	do.	do.	8.1	+	6.9	7.16
147	.....	do.	do.	8.8	+	6.8	7.06
148	.....	do.	do.	10.3	+	7.3	7.69
149	.....	do.	do.	8.3	+	7.1	7.62
150	.....	do.	do.	6.4	+	7.3	7.65
151	.....	do.	do.	12.3	+	6.0	6.25
152	.....	do.	do.	9.4	+	6.0	5.96

[a] One sample.

TABLE VII.—*Type of growth, nitrogen fixed, and reaction of soils of Series II*—Continued

Soil No.	Duplicate soil.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
				Mgm.			
153	.....	Nontypical Azotobacter.	Azotobacter present.	2.8	−	5.6	5.59
154	.....	Typical Azotobacter.	Typical Azotobacter.	8.3	+	6.0	6.17
155	.....	....do.....	....do.....	9.7	+	6.1	6.34
156	.....	None.....	No Azotobacter.....	9.6	−	5.5	5.61
157	.....	Typical Azotobacter.	Typical Azotobacter.	8.0	+	6.0	6.27
158	.....	Nontypical Azotobacter.	Azotobacter present.	8.1	+	6.2	6.51
159	.....	Typical Azotobacter.	Typical Azotobacter.	8.3	+	7.8	7.77
160	.....	....do.....	....do.....	10.1	+	6.2	6.89

TABLE VIII.—*Correlation between reaction and presence of Azotobacter in soils of Series II*

P_H determined electrometrically.						P_H determined colorimetrically.					
Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.
143	8.77	+	151	6.25	+	143	8.1	+	155	6.1	+
145	7.94	+	154	6.17	+	142	7.8	+	116	6.0	+
159	7.77	+	152	5.96	+	159	7.8	+	151	6.0	+
144	7.72	+	139	5.90	+	129	7.6	+	152	6.0	+
107	7.70	+	116	5.90	+	118	7.5	+	154	6.0	+
148	7.69	+	113	5.85	+	107	7.4	+	157	6.0	+
150	7.65	+	128	5.78	−	110	7.4	+	123	5.9	+
149	7.62	+	120	5.68	+	130	7.4	+	128	5.9	−
110	7.61	+	123	5.64	+	133	7.3	+	132	5.9	+
142	7.59	+	156	5.61	−	144	7.3	+	112	5.8	+
130	7.52	+	153	5.59	−	148	7.3	+	114	5.8	+
141	7.48	+	114	5.59	+	150	7.3	+	120	5.8	+
133	7.45	+	137	5.58	−	105	7.2	+	122	5.8	+
129	7.30	+	132	5.54	+	135	7.2	+	131	5.8	−
140	7.30	+	122	5.51	+	141	7.2	+	137	5.8	−
118	7.28	+	115	5.51	+	145	7.2	+	106	5.7	−
135	7.20	+	112	5.48	+	104[a]	7.1	+	121	5.7	−
146	7.16	+	108	5.48	+	119	7.1	+	108	5.6	+
105	7.08	+	131	5.47	−	149	7.1	+	113	5.6	+
124	7.06	+	103	5.27	−	140	7.0	+	153	5.6	−
147	7.06	+	106	5.25	−	124	6.9	+	125	5.5	−
119	7.00	+	121	5.24	−	146	6.9	+	126	5.5	−
160	6.89	+	126	5.19	−	147	6.8	+	127	5.5	−
101	6.71	+	125	5.15	−	101	6.7	+	156	5.5	−
117	6.56	+	127	5.10	−	109	6.6	+	103	5.4	−
158	6.51	+	138	5.05	−	111	6.6	+	115	5.4	+
109	6.47	+	134	4.88	−	117	6.6	+	134	5.4	−
111	6.39	+	136	4.88	+	139	6.2	+	136	5.4	+
155	6.34	+	102	3.78	−	158	6.2	+	138	5.4	−
157	6.27	+	.....	.....	.....	160	6.2	+	102	5.0	−

[a] P_H not determined electrometrically.

In the following summary it may be observed that there were no soils more alkaline than P_H 6.0 that did not contain Azotobacter. There were 13 soils with an electrometric and 10 with a colorimetric P_H below 6.0 that were recorded as containing Azotobacter. Only 5 of these produced typical Azotobacter film and 10 of the 13 gave a P_H of 5.8 or above by one of the methods. It would appear from the data of this series of soils that P_H 5.8 was nearer the absolute limiting hydrogen-ion concentration than P_H 6.0.

Summary of Tables VII and VIII

Number of soils examined	60
Number of soils containing Azotobacter	46
Number of soils not containing Azotobacter	14
Average mgm. nitrogen fixed, 59 soils	8.41
Average mgm. nitrogen fixed, 45 soils containing Azotobacter	9.16
Average mgm. nitrogen fixed, 14 soils not containing Azotobacter	6.03
Number of soils electrometric P_H 6.0 or above	33
Number of soils electrometric P_H below 6.0	27
Number of soils colorimetric P_H 6.0 or above	36
Number of soils colorimetric P_H below 6.0	24
Number of soils electrometric P_H 6.0 or above containing Azotobacter	33
Number of soils electrometric P_H 6.0 or above not containing Azotobacter	0
Number of soils electrometric P_H below 6.0 containing Azotobacter	13
Number of soils electrometric P_H below 6.0 not containing Azotobacter	14
Number of soils colorimetric P_H 6.0 or above containing Azotobacter	36
Number of soils colorimetric P_H 6.0 or above not containing Azotobacter	0
Number of soils colorimetric P_H below 6.0 containing Azotobacter	10
Number of soils colorimetric P_H below 6.0 not containing Azotobacter	14
Average electrometric P_H, 59 soils	6.37
Average colorimetric P_H, 60 soils	6.39
Average electrometric P_H, 45 soils containing Azotobacter	6.73
Average electrometric P_H, 14 soils not containing Azotobacter	5.21
Average colorimetric P_H, 46 soils containing Azotobacter	6.65
Average colorimetric P_H, 14 soils not containing Azotobacter	5.55
Association coefficient based on electrometric P_H determinations	1.000
Association coefficient based on colorimetric P_H determinations	1.000

The above summary with a few minor exceptions is very similar to that for Series I. The most marked difference is in the average nitrogen fixed by the soils not containing Azotobacter, this being 3.86 mgm. for Series I and 6.03 mgm. for Series II. This difference is due to the much higher fixation of nitrogen by soils not containing Azotobacter in media containing calcium carbonate than in the media without calcium carbonate. This fact has been emphasized in a former article (*4*). No calcium carbonate was added to the media employed in the examination of the soils of Series I, while an excess was added to the media in the examination of all subsequent soils.

SERIES III

The soils of Series III were secured through the county agents of Kansas and did not reach the laboratory in as fresh and possibly as uncontaminated condition as did those collected locally. Samples 138 to 180, inclusive, reached the laboratory while the writer was in the Army and remained several months before being examined. It was believed that the quantity of nitrogen fixed by these soils would not be comparable to that of fresh soils, hence no nitrogen determinations were made. Possibly these soils should be disregarded entirely. The data secured from the examination of the soils of this series are recorded in Tables IX and X, and summarized below.

TABLE IX.—*Type of growth, nitrogen fixed, and reaction of soils of Series III*

Soil No.	County.	Type of film.	Microscopic picture	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
				Mgm.			
161	Pottawatamie.	Typical Azotobacter.	Typical Azotobacter.	8.6	+	6.5	7.21
162	do.	Nontypical film.	Azotobacter (?)	7.1	+	5.7	6.02
163	Cloud.	do.	do.	5.8	+	6.0	5.98
164	do.	None.	No Azotobacter.	5.0	−	5.7	5.41
165	do.	do.	do.	4.4	−	5.6	5.54
166	do.	do.	do.	7.0	−	5.5	5.71
167	Wyandotte.	Nontypical.	Typical Azotobacter.	8.0	+	6.1	6.25
168	Franklin.	None.	No Azotobacter.	6.3	−	5.4	5.17
169	do.	Nontypical.	Azotobacter present.	7.1	+	5.2	5.10
170	Wilson.	do.	do.	2.9	+	5.9	5.93
171	do.	do.	do.	2.9	+	5.4	5.15
172	do.	None.	No Azotobacter.	5.1	−	5.3	5.02
173	Pawnee.	Typical Azotobacter.[a]	Typical Azotobacter.[a]	8.2	+	6.0	6.08
174	Franklin.	None.	Azotobacter (?)	5.4	−	5.8	5.85
175	do.	do.	do.	3.0	−	5.4	5.29
176	Anderson.	Typical Azotobacter.	Typical Azotobacter.	9.1	+	6.5	6.35
177	do.	None.	Azotobacter (?)	7.2	−	5.6	5.37
178	do.	do.	No Azotobacter.	9.6	−	5.6	5.30
179	Nemaha.	do.	do.	4.7	−	5.8	5.76
180	do.	do.	do.	1.8	−	5.6	5.47
181	Marshall.	Nontypical.	Azotobacter (?)	7.9	+	5.9	6.10
182	do.	None.	No Azotobacter.	7.8	−	6.2	6.52
183	do.	Typical Azotobacter.	Typical Azotobacter.	8.8	+	6.9	7.37
184	do.	do.	do.	8.5	+	6.4	6.77
185	Wyandotte.	None.	No Azotobacter.	4.6	−	5.6	5.44
186	do.	Typical Azotobacter.	Typical Azotobacter.	9.9	+	7.2	7.72
187	do.	do.	do.	8.5	+	6.2	6.69
188	Atchinson.	Nontypical.	Azotobacter present.	7.4	+	5.6	5.52
189	do.	None.	No Azotobacter.	0.8	−	5.6	5.52
190	do.	do.	Azotobacter present.[a]	5.5	−	5.6	5.34
191	do.	do.	No Azotobacter.	5.3	−	5.7	5.32
192	Wilson.	do.	do.	7.2	−	5.6	5.22
193	Jewell.	Nontypical.	Typical Azotobacter.	9.5	+	6.6	6.84
194	do.	Typical Azotobacter.	do.	8.1	+	6.0	6.15
195	Chase.	None.	No Azotobacter.	7.7	−	5.8	5.69
196	do.	do.	do.	6.6	−	5.4	4.90
197	do.	Typical Azotobacter.	Typical Azotobacter.	6.9	+	6.6	7.57
198	Lyon.	None.	Azotobacter (?)	4.6	−	5.9	5.49
199	do.	Nontypical.	Typical Azotobacter.	5.1	+	5.9	6.07
200	Osborne.	Typical Azotobacter.	do.	7.9	+	7.6	8.28
201	do.	None.	No Azotobacter.	6.1	−	6.1	5.98
202	Kearney.	Typical Azotobacter.	Typical Azotobacter.	7.5	+	7.4	8.19
203	do.	do.	do.	7.0	+	7.0	7.25

[a] One sample.

TABLE IX.—*Type of growth, nitrogen fixed, and reaction of soils of Series III*—Contd.

Soil No.	County.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
				Mgm.			
204	Washington	None	No Azotobacter	2.7	−	5.8	5.10
205	Doniphan	Typical Azotobacter.	Typical Azotobacter.	7.9	+	6.1	6.02
206	do	do.[a]	do.[a]	5.4	−	5.7	5.32
207	do	do	do	.....	+	7.1	7.94
208	Rush	do	do	11.6	+	7.2	7.60
209	Greenwood	None	No Azotobacter	0.3	−	5.7	5.53
210	do	do	do	5.7	−	5.3	5.05
211	do	do	do	4.0	−	5.3	5.10
212	do	do	do	5.7	−	5.6	5.39
213	Rawlins	Typical Azotobacter.	Typical Azotobacter.	10.5	+	7.6	8.30
214	do	do	do	10.0	+	7.3	7.98
215	do	do	do	9.3	+	6.7	7.28
216	do	do	do	9.6	+	6.9	7.40
217	Greenwood	do	do	11.1	+	5.6	5.15
218	Jewell	do	do	10.6	+	6.6	7.28
219	do	None	No Azotobacter	6.0	−	6.6	7.25
220	Pottawatamie.	Typical Azotobacter.	Typical Azotobacter.	11.2	+	6.4	6.34
221	Ford	do.[a]	do.[a]	8.2	+	6.6	6.66
222	do	do.[a]	do.[a]	11.8	+	6.5	6.69
223	do	do	do	9.5	+	6.7	6.63
224	do	do	do	9.3	+	6.9	7.52
225	McPherson	Nontypical	do	7.4	+	5.9	5.91
226	do	Typical Azotobacter.	do	3.6	+	6.7	7.13
227	do	None	No Azotobacter	5.0	−	5.8	5.66
228	do	do	do	5.1	−	5.6	5.20
229	Burbon	Typical Azotobacter.	Typical Azotobacter.	10.2	+	7.1	7.60
230	Osborne	do	do	8.9	+	6.6	6.98
231	Kearney	Nontypical	do	7.0	+	7.4	8.25
232	do	Typical Azotobacter.	do	10.1	+	7.4	8.31
233	Greenwood	Nontypical	do	7.0	+	7.0	7.47
234	Meade	Typical Azotobacter.	do	8.1	+	7.4	8.15
235	do	do	do	8.7	+	6.5	6.66
236	Chautauqua	None	No Azotobacter	4.5	−	5.6	5.30
237	do	do	Azotobacter (?)	3.7	−	5.5	5.02
238	Morris	do	No Azotobacter	.....	−	5.7	5.54
239	Pawnee	Typical Azotobacter.	Typical Azotobacter.	.....	+	6.1	6.08
240	do	do	do	.....	+	6.6	7.03
241	Meade	Nontypical	do	.....	+	6.4	6.18
242	Elk	do.[a]	do.[a]	.....	−	5.9	5.51
243	Meade	None	No Azotobacter	.....	−	6.7	6.96
244	Chautauqua	do	Azotobacter (?)	.....	−	5.1	5.22
245	Rush	do	do	.....	−	6.1	6.54
246	Douglas	do	No Azotobacter	.....	−	5.8	5.90
247	do	do	do	.....	−	5.5	5.37
248	do	do	do	.....	−	5.7	5.47
249	do	do	do	.....	−	5.4	5.12
250	Coffey	Nontypical	Typical Azotobacter.	.....	+	5.9	6.00
251	do	None	No Azotobacter	.....	−	6.0	6.10

[a] One sample.

TABLE IX.—*Type of growth, nitrogen fixed, and reaction of soils of Series III*—Contd.

Soil No.	County.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
				Mgm.			
252..	Coffey	None.....	No Azotobacter..	.....	−	5.6	5.37
253..	Pawnee.....	Typical Azotobacter.	Typical Azotobacter.	.....	+	6.4	6.25
254..	Greenwood .	None.....	No Azotobacter..	.....	−	5.4	4.68
255..	Wichita....	Typical Azotobacter.	Typical Azotobacter.	.....	+	7.2	7.79
256..	.....do.....	None.....	Azotobacter (?)...	.....	−	6.2	6.35
257..	.....do.....	Nontypical.....	Typical Azotobacter	.....	+	7.8	8.13
258..	.....do.....	Typical Azotobacter.	.....do.....	.....	+	7.6	8.26
259..	Logan.....	.....do..... ...	.....do.....	.....	+	6.5	6.49
260..	Thomas.....	None.....	Azotobacter (?)...	.....	−	7.0	7.48
261..	Pratt.....	.....do.....	No Azotobacter..	.....	−	5.9	5.81
262..	...do.....	.....do.....	.....do.....	.....	−	6.9	7.23
263..	Lyon.....	.....do.....	Azotobacter (?)...	.....	−	5.5	4.93
264..	Hodgeman..	Typical Azotobacter.	Typical Azotobacter.	.....	+	7.4	7.52
271..	Woodson....	None.....	No Azotobacter...	.....	−	5.6	5.41
272..	Pratt.....	Typical Azotobacter.	Typical Azotobacter.	.....	+	7.4	7.72
273..	.....do.....	None.....	No Azotobacter..	.....	−	6.5	6.42
274..	Finney.....	Nontypical.....	Typical Azotobacter.	.....	+	6.5	6.96
275..	.....do.....	Typical Azotobacter.	.....do.....	.....	+	7.4	8.06
276..	Jackson.....	None.....	No Azotobacter..	.....	−	5.7	5.27
277..	.....do.....	.....do.....	.....do.....	.....	−	5.4	5.10
278..	Hodgeman..	Nontypical.....	Azotobacter (?)...	.....	+	7.4	7.99
279..	.....do.....	None.....	No Azotobacter..	.....	−	6.4	6.81
280..	.....do.....	Typical Azotobacter.	Typical Azotobacter.	.....	+	5.7	6.51

TABLE X.—*Correlation between reaction and presence of Azotobacter in soils of Series III*

PH determined electrometrically.						PH determined colorimetrically.					
Soil No.	PH.	Azotobacter.	Soil No.	PH.	Azotobacter.	Soil No.	PH.	Azotobacter.	Soil No.	PH.	Azotobacter.
232...	8.31	+	239...	6.08	+	257...	7.8	+	173...	6.0	+
213...	8.30	+	173...	6.08	+	200...	7.6	+	194...	6.0	+
200...	8.28	+	199...	6.07	+	213...	7.6	+	251...	6.0	−
258...	8.26	+	162...	6.02	+	258...	7.6	+	170...	5.9	+
231...	8.25	+	205...	6.02	+	202...	7.4	+	181...	5.9	+
202...	8.19	+	250...	6.00	+	231...	7.4	+	198...	5.9	−
234...	8.15	+	201...	5.98	−	232...	7.4	+	199...	5.9	+
257...	8.13	+	163...	5.98	+	234...	7.4	+	225...	5.9	+
275...	8.06	+	170...	5.93	+	264...	7.4	+	242...	5.9	−
278...	7.99	+	225...	5.91	+.	272...	7.4	+	250...	5.9	+
214...	7.98	+	246...	5.90	−	275...	7.4	+	261...	5.9	−
207...	7.94	+	174...	5.85	−	278...	7.4	+	174...	5.8	−
255...	7.79	+	261...	5.81	−	214...	7.3	+	179...	5.8	−
272...	7.72	+	195...	5.80	−	186...	7.2	+	195...	5.8	−
186...	7.72	+	179...	5.76	−	208...	7.2	+	204...	5.8	−
208...	7.60	+	166...	5.71	−	255...	7.2	+	227...	5.8	−
229...	7.60	+	227...	5.66	−	207...	7.1	+	246...	5.8	−
197...	7.57	+	238...	5.54	−	229...	7.1	+	162...	5.7	+
264...	7.52	+	165...	5.54	−	203...	7.0	+	164...	5.7	−
224...	7.52	+	209...	5.53	−	233...	7.0	+	191...	5.7	−
260...	7.48	−	188...	5.52	+	260...	7.0	−	206...	5.7	−
233...	7.47	+	189...	5.52	−	183...	6.9	+	209...	5.7	−
216...	7.40	+	242...	5.51	−	216...	6.9	+	238...	5.7	−
183...	7.37	+	198...	5.49	−	224...	6.9	+	248...	5.7	−
218...	7.28	+	248...	5.47	−	262...	6.9	−	276...	5.7	−
215...	7.28	+	180...	5.47	−	215...	6.7	+	280...	5.7	+
219...	7.25	−	185...	5.44	−	223...	6.7	+	165...	5.6	−
203...	7.25	+	164...	5.41	−	226...	6.7	+	177...	5.6	−
262...	7.23	−	271...	5.41	−	243...	6.7	−	178...	5.6	−
161...	7.21	+	212...	5.39	−	193...	6.6	+	180...	5.6	−
226...	7.13	+	177...	5.37	−	197...	6.6	+	185...	5.6	−
240...	7.03	+	252...	5.37	−	218...	6.6	+	188...	5.6	+
230...	6.98	+	247...	5.37	−	219...	6.6	−	189...	5.6	−
243...	6.96	−	190...	5.34	−	221...	6.6	+	190...	5.6	−
274...	6.96	+	206...	5.32	−	230...	6.6	+	192...	5.6	−
182...	6.90	−	191...	5.32	−	240...	6.6	+	212...	5.6	−
193...	6.84	+	236...	5.30	−	161...	6.5	+	217...	5.6	+
279...	6.81	−	178...	5.30	−	176...	6.5	+	228...	5.6	−
184...	6.77	+	175...	5.29	−	222...	6.5	+	236...	5.6	−
222...	6.69	+	276...	5.27	−	235...	6.5	+	252...	5.6	−
187...	6.69	+	192...	5.22	−	259...	6.5	+	271...	5.6	−
235...	6.66	+	244...	5.22	−	273...	6.5	−	166...	5.5	−
221...	6.66	+	228...	5.20	−	274...	6.5	+	237...	5.5	−
223...	6.63	+	168...	5.17	−	184...	6.4	+	263...	5.5	−
245...	6.54	−	217...	5.15	+	220...	6.4	+	247...	5.5	−
280...	6.51	+	171...	5.15	+	241...	6.4	+	168...	5.4	−
259...	6.49	+	249...	5.12	−	253...	6.4	+	171...	5.4	+
273...	6.42	−	277...	5.10	−	279...	6.4	−	175...	5.4	−
256...	6.35	−	204...	5.10	−	182...	6.2	−	196...	5.4	−
176...	6.35	+	211...	5.10	−	187...	6.2	+	249...	5.4	−
220...	6.34	+	169...	5.10	+	256...	6.2	−	254...	5.4	−
253...	6.25	+	210...	5.05	−	167...	6.1	+	277...	5.4	−
167...	6.25	+	237...	5.02	−	201...	6.1	−	172...	5.3	−
241...	6.18	+	172...	5.02	−	205...	6.1	+	210...	5.3	−
194...	6.15	+	263...	4.93	−	239...	6.1	+	211...	5.3	−
251...	6.10	−	196...	4.90	−	245...	6.1	−	169...	5.2	+
181...	6.10	+	254...	4.68	−	163...	6.0	+	244...	5.1	−

Summary of Tables IX and X

Number of soils examined	114
Number of soils containing Azotobacter	60
Number of soils not containing Azotobacter	54
Average mgm. nitrogen fixed, 76 soils	6.95
Average mgm. nitrogen fixed, 44 soils containing Azotobacter	8.28
Average mgm. nitrogen fixed, 32 soils not containing Azotobacter	5.10
Number of soils electrometric P_H 6.0 or above	63
Number of soils electrometric P_H below 6.0	51
Number of soils colorimetric P_H 6.0 or above	60
Number of soils colorimetric P_H below 6.0	54
Number of soils electrometric P_H 6.0 or above containing Azotobacter	53
Number of soils electrometric P_H 6.0 or above not containing Azotobacter	10
Number of soils electrometric P_H below 6.0 containing Azotobacter	7
Number of soils electrometric P_H below 6.0 not containing Azotobacter	44
Number of soils colorimetric P_H 6.0 or above containing Azotobacter	49
Number of soils colorimetric P_H 6.0 or above not containing Azotobacter	11
Number of soils colorimetric P_H below 6.0 containing Azotobacter	11
Number of soils colorimetric P_H below 6.0 not containing Azotobacter	43
Average electrometric P_H, 114 soils	6.32
Average colorimetric P_H, 114 soils	6.21
Average electrometric P_H, 60 soils containing Azotobacter	6.94
Average electrometric P_H, 54 soils not containing Azotobacter	5.63
Average colorimetric P_H, 60 soils containing Azotobacter	6.62
Average colorimetric P_H, 54 soils not containing Azotobacter	5.77
Association coefficient based on electrometric P_H determinations	0.942
Association coefficient based on colorimetric P_H determinations	0.891

The data here summarized vary but slightly from those for the two former series. There were 10 soils with an electrometric and 11 with a colorimetric P_H of 6.0 or above that failed to show Azotobacter. Of these 11, 8 were among those that remained in the laboratory for several months before being examined. One of the remaining 3 reacted more acid and 1 only slightly less acid than P_H 6.0 by one of the methods.

There were 7 soils that reacted electrometrically and 11 colorimetrically more acid than P_H 6.0, recorded as containing Azotobacter. Six of these reacted less acid than P_H 6.0 and 2 of the remaining less acid than P_H 5.9 by one of the methods. Only 1 soil more acid than P_H 5.9 produced a typical Azotobacter growth accompanied by good fixation of nitrogen.

SERIES IV

In Table XI and XII data collected from the soils of Series IV are presented. These soils were collected in a large number of instances from experimental plots where the effect of lime upon acid soils was being studied. Such plots are usually located upon areas of acid soil. From observation such soils do not contain Azotobacter. The data here presented show that many of these limed soils should be, as far as reaction is concerned, favorable to the growth of Azotobacter, yet no Azotobacter were found. In other words, the addition of lime has changed the hydrogen-ion from an unfavorable to a favorable concentration for Azotobacter without apparently influencing the Azotobacter flora. There are many limed soils, however, to which the quantity of lime added apparently has not been sufficient to change appreciably the hydrogen-ion concentration.

TABLE XI.—*Type of growth, nitrogen fixed, and reaction of soils from different States, Series IV*

Soil No.	State.	Limed.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
					Mgm.			
301	Michigan...	o	None.....	No Azotobacter.	4.2	−	5.9	5.76
302	.....do.....	o	Typical Azotobacter.	Typical Azotobacter.	7.1	+	5.6	6.64
303	.....do.....	o	None.....	No Azotobacter.	3.7	−	7.4	7.38
304	.....do.....	o	Typical Azotobacter.	Typical Azotobacter.	11.4	+	6.3	5.73
305	.....do.....	o	None.....	No Azotobacter.	4.8	−	5.9	5.69
306	California..	o	Nontypical...	Typical Azotobacter.	8.1	+	6.1	5.61
307	.....do.....	o	None.....	No Azotobacter.	6.5	−	5.9	5.49
308	.....do.....	o	.....do.....	Typical Azotobacter.[a]	5.6	−	6.3	5.81
309	.....do.....	o	Typical Azotobacter.	.....do.....	10.8	+	7.8	8.14
310	.....do.....	o	None.....	.....do.....	5.4	+	6.3	7.08
311	Ohio.....	+	.....do.....	No Azotobacter.	0.3	−	6.6	7.01
312	.....do.....	+	.....do.....	.....do.....	3.6	−	7.0	7.33
313	.....do.....	+	.....do.....	.....do.....	4.7	−	4.8	5.05
314	.....do.....	o	.....do.....	.....do.....	4.8	−	5.9	6.03
315	.....do.....	o	.....do.....	.....do.....	4.3	−	5.9	6.07
316	North Carolina.	o	.....do.....	.....do.....	4.5	−	4.6	5.00
317	.....do.....	+	.....do.....	.....do.....	6.0	−	6.4	6.78
318	.....do.....	+	.....do.....	.....do.....	5.0	−	5.9	6.01
319	New Jersey.	+	.....do.....	Azotobacter (?)	5.3	−	6.8	6.71
320	.....do.....	o	.....do.....	Azotobacter present.	4.8	−	4.9	4.80
321	.....do.....	+	.....do.....	Azotobacter (?)	6.0	−	6.4	6.52
322	.....do.....	+	.....do.....	Azotobacter present.	4.4	−	7.0	6.93
323	.....do.....	o	.....do.....	No Azotobacter.	4.4	−	4.9	4.90
324	Iowa.....	o	Typical Azotobacter.	Typical Azotobacter.	11.6	+	7.0	7.11
325	.....do.....	o	None.....	No Azotobacter.	4.9	−	4.9	4.78
326	.....do.....	o	.....do.....	.....do.....	4.8	−	4.8	4.83
327	.....do.....	o	Typical Azotobacter.[a]	Typical Azotobacter.[a]	6.5	+	4.9	4.93
328	.....do.....	o	None.....	No Azotobacter.	4.5	−	5.3	5.88
329	New York..	+	Typical Azotobacter.	Typical Azotobacter.	7.5	+	7.2	7.20
330	.....do.....	+	None.....	.....do.[a].....	5.5	+	7.0	6.84
331	.....do.....	+	Nontypical...	.....do.....	6.6	+	6.9	6.79
332	.....do.....	+	None.....	Azotobacter (?)	6.5	−	6.5	6.23
333	.....do.....	o	.....do.....	.....do. (?)....	5.7	−	5.2	5.47
334	Arkansas....	o	.....do.....	.....do. (?)....	7.8	+	7.0	6.96
335	.....do.....	o	.....do.....	.....do. (?)....	5.4	−	4.9	5.05
336	.....do.....	o	.....do.....	.....do. (?)....	5.0	−	5.5	5.58
337	Oregon....	o	.....do.....	No Azotobacter.	2.7	−	5.2	5.27
338	.....do.....	o	.....do.....	.....do.....	3.5	−	5.4	5.39
339	.....do.....	o	.....do.....	.....do.....	3.3	−	5.0	5.24
340	.....do.....	o	.....do.....	.....do.....	3.1	−	5.6	5.93

[a] One sample only.

TABLE XI.—*Type of growth, nitrogen fixed, and reaction of soils from different States, Series IV*—Continued.

Soil No.	State.	Limed.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
					Mgm.			
341	Oregon	o	None	Azotobacter	3.5	–	5.5	5.66
342	Alabama	o	do	do	1.0	–	4.6	4.44
343	do	o	do	do	1.6	–	5.9	5.83
344	do	o	do	do	0.3	–	4.8	5.05
345	do	+	do	Azotobacter (?)	3.0	–	5.9	5.66
346	do	+	do	No Azotobacter.	1.7	–	5.9	5.51
347	Mississippi	o	Typical Azotobacter.	Typical Azotobacter.	8.0	+	6.2	6.17
348	do	o	None	Azotobacter (?)	2.5	–	4.6	4.21
349	do	o	Typical Azotobacter.	Typical Azotobacter.	8.3	+	6.6	6.76
350	do	o	do	do	8.6	+	6.2	6.20
351	do	o	do	do	9.5	+	7.4	7.74
352	Illinois	+	do	do	.....	+	6.3	7.20
353	do	o	None	No Azotobacter.	.....	–	4.8	5.00
354	do	+	Typical Azotobacter.	Typical Azotobacter.	.....	+	5.8	6.51
355	do	o	None	No Azotobacter.	.....	–	4.6	4.19
356	do	o	do	do	.....	–	4.7	4.70
357	do	+	do	do	.....	–	5.2	5.73
358	Indiana	o	do	do	4.0	–	4.3	3.80
359	do	+	do	do	6.0	–	5.0	4.93
360	Georgia	+	do	do	5.8	–	6.6	6.69
361	do	+	do	do	6.1	–	5.8	6.05
362	do	o	do	do	6.6	–	5.0	5.24
363	do	o	do	do	6.6	–	6.1	5.85
364	do	o	do	do	5.8	–	4.8	4.76
365	Ohio	+	do	do	6.2	–	6.2	5 88
366	do	+	Nontypical	Typical Azotobacter.	6.8	+	6.8	6.84
367	do	+	None	No Azotobacter.	5.1	–	6.2	6.18
368	do	o	Nontypical	Typical Azotobacter.	9.0	+	7.1	7.48
369	do	o	do	do	10.4	+	6.7	7.05
370	do	o	None	Azotobacter present.	9.6	+	7.0	7.45
371	New Jersey	o	do	No Azotobacter.	5.3	–	4.8	4.70
372	do	+	do	do	5.1	–	5.7	5.37
373	do	o	do	do	4.7	–	5.0	4.22
374	do	+	do	do	5.3	–	6.5	6.44
375	do	o	do	do	5.0	–	4.8	5.12
376	do	+	do	do	5.4	–	6.8	6.86
377	North Carolina.	o	do	do	5.2	–	5.0	4.98
378	do	o	Nontypical[a]	Typical Azotobacter.[a]	5.4	–	4.8	4.90
379	do	o	None	No Azotobacter.	5.4	–	4.4	3.73
380	do	o	do	do	5.2	–	5.0	4.93
381	do	o	do	do	4.4	–	5.4	5.09
382	do	o	do	do	5.8	–	5.0	4.63
383	Massachusetts.	+	Nontypical	Azotobacter (?)	4.0	–	6.5	6.29
384	do	+	do	do.(?)	4.3	–	6.6	6.27

[a] One sample only.

TABLE XI—*Type of growth, nitrogen fixed, and reaction of soils of different States, Series IV*—Continued.

Soil No.	State.	Limed.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
					Mgm.			
385	Massachusetts.	+	None	No Azotobacter.	4.0	−	5.7	5.31
386	do	+	do	do	4.1	−	5.6	5.24
387	do	o	do	do	4.4	−	4.4	4.10
388	do	o	do	do	3.5	−	4.6	4.01
389	Maryland	o	do	do	6.4	−	4.8	4.70
390	do	+	do	do	4.0	−	4.8	4.90
391	do	+	do	do	4.2	−	6.6	6.77
392	do	o	do	do	5.2	−	4.7	4.48
393	do	o	do	do	4.5	−	4.7	4.51
394	do	+	do	do	4.4	−	6.0	6.18
395	Rhode Island.	o	do	do	4.6	−	4.7	4.58
396	do	+	do	do	2.7	−	5.6	5.64
397	do	o	do	do	3.9	−	4.9	5.30
398	do	+	do	do	4.6	−	6.6	6.77
399	do	o	do	do	3.8	−	6.5	5.88
400	do	+	do	do	3.9	−	4.6	4.31
401	Virginia	+	Typical Azotobacter.	Typical Azotobacter.	6.0	+	5.6	6.15
402	do	+	do	do	6.8	+	6.7	7.03
403	do	+	do	do	6.8	+	7.0	7.30
404	do	o	None	No Azotobacter.	4.2	−	5.0	4.70
405	do	o	do	do	5.8	−	4.9	4.75
406	do	o	do	do	1.3	−	5.2	4.83
407	Kentucky	o	do	do	6.6	−	4.9	4.82
408	do	+	do	do	4.8	−	6.2	6.12
409	do	o	do	do	4.2	−	5.8	5.19
410	do	+	do	do	5.2	−	6.8	7.06
411	do	o	do	do	7.0	−	4.8	4.82
412	do	+	do	do	5.4	−	6.7	6.62
413	North Carolina.	o	do	do	3.9	−	6.0	6.28
414	do	+	do	do	4.3	−	5.2	4.97
415	do	o	do	do	3.9	−	4.9	4.81
416	do	+	do	do	2.8	−	5.9	6.07
417	do	o	do	do	3.5	−	4.8	4.66
418	Tennessee	o	Nontypical	Typical Azotobacter.	3.2	+	4.9	4.68
419	do	+	do	do	2.5	+	5.5	5.69
420	do	o	None	Azotobacter (?)	1.0	−	4.8	4.59
421	do	+	Nontypical	Typical Azotobacter.	4.0	+	5.4	5.42
422	do	o	do	do	3.2	+	4.8	4.53
423	do	+	do	do	5.4	+	5.5	5.68
424	West Virginia.	o	None	No Azotobacter.	4.8	−	4.6	4.09
425	do	+	do	do	6.6	−	4.8	4.66
426	do	o	do	do	4.2	−	4.6	4.02
427	do	+	do	do	4.1	−	5.0	4.90
428	do	o	do	do	3.3	−	4.6	3.99
429	do	+	do	do	4.6	−	5.5	5.51
430	Indiana	o	do	do	5.7	−	4.8	4.58
431	do	+	Typical Azotobacter.[a]	Typical Azotobacter.[a]	8.3	+	5.6	5.03
432	New York	+	do	do	6.9	+	6.7	7.30
433	do	+	Nontypical[a]	Azotobacter present.	5.1	−	6.6	6.78

[a] One sample only.

TABLE XI.—*Type of growth, nitrogen fixed, and reaction of soils from different States, Series IV*—Continued.

Soil No.	State.	Limed.	Type of film.	Microscopic picture.	Nitrogen fixed.	Azotobacter.	P_H colorimetric.	P_H electrometric.
					Mgm.			
434	New York..	+	Nontypical..	Typical Azotobacter.	6.1	+	6.2	6.84
435	.....do.....	+	None.....	Azotobacter (?)	7.3	−	5.7	6.23
436	.....do.....	o	.....do.....	No Azotobacter.	4.4	−	5.0	4.98
437	.....do.....	+	Typical Azotobacter.	Typical Azotobacter.	8.3	+	6.3	6.72
438	South Carolina.	o	Nontypical...	.....do.....	6.4	+	5.1	4.98
439	.....do.....	o	Typical Azotobacter.	.....do.....	8.9	+	7.6	8.14
440	.....do.....	+	.....do.....	.....do.....	6.8	+	6.8	7.84
441	.....do.....	o	Nontypical [a].	.....do.....	5.3	−	4.9	4.90
442	.....do.....	+	None.....	No Azotobacter.	4.2	−	4.9	5.15
443	.....do.....	+	.....do.....	.....do.....	4.4	−	5.2	5.34
444	Pennsylvania.	o	Typical Azotobacter.	Typical Azotobacter.	7.6	+	7.0	7.20
445	.....do.....	o	None.....	No Azotobacter.	5.6	−	5.2	5.59
446	.....do.....	o	.....do.....	Azotobacter (?)	6.3	−	4.8	5.07
447	.....do.....	+	Typical Azotobacter.	Typical Azotobacter.	8.0	+	7.4	8.41
448	.....do.....	+	.....do.....	.....do.....	8.1	+	8.2	8.25
449	.....do.....	o	None.....	No Azotobacter.	4.7	−	4.6	4.24
265	Missouri....	o	.....do.....	.....do.....	5.0	−	4.7	4.36
266	.....do.....	o	.....do.....	.....do.....	4.5	−	5.2	4.97
267	.....do.....	+	Typical Azotobacter	Typical Azotobacter.	5.8	+	6.9	7.10
268	.....do.....	o	.....do.....	.....do.....	9.1	+	7.1	7.67
269	.....do.....	o	.....do.....	.....do.....	7.5	+	6.7	7.18

[a] One sample only.

TABLE XII.—*Correlation between reaction and presence of Azotobacter in soils of Series IV*

P_H determined electrometrically.						P_H determined colorimetrically.					
Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.
447...	8.41	+	445...	5.59	−	448...	8.2	+	401...	5.6	+
448...	8.25	+	336...	5.58	−	309...	7.8	+	431...	5.6	+
439...	8.14	+	429...	5.51	−	439...	7.6	+	429...	5.5	−
309...	8.14	+	346...	5.51	−	303...	7.4	−	336...	5.5	−
440...	7.84	+	307...	5.49	−	351...	7.4	+	341...	5.5	−
366...	7.84	+	333...	5.47	−	447...	7.4	+	419...	5.5	+
391...	7.77	−L [a]	421...	5.42	+	329...	7.2	+	423...	5.5	+
351...	7.74	+	338...	5.39	−	368...	7.1	+	338...	5.5	−
268...	7.67	+	372...	5.37	−	268...	7.1	+	381...	5.4	−
368...	7.48	+	443...	5.34	−	312...	7.0	−L	421...	5.4	+
370...	7.45	+	385...	5.31	−	322...	7.0	−L	328...	5.3	−
303...	7.38	−	397...	5.30	−	324...	7.0	+	443...	5.2	−
312...	7.33	−L	337...	5.27	−	330...	7.0	+	445...	5.2	−
403...	7.30	+	386...	5.24	−	334...	7.0	+	226...	5.2	−
432...	7.30	+	339...	5.24	−	370...	7.0	+	333...	5.2	−
352...	7.20	+	362...	5.24	−	403...	7.0	+	337...	5.2	−
444...	7.20	+	409...	5.19	−	444...	7.0	+	357...	5.2	−

[a] L, Limed soil.

TABLE XI.—*Type of growth, nitrogen fixed, and reaction of soils from different States, Series IV*—Continued.

P_H determined electrometrically.						P_H determined colorimetrically.					
Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.	Soil No.	P_H.	Azotobacter.
329...	7.20	+	442...	5.15	−	331...	6.9	+	406...	5.2	−
269...	7.18	+	375...	5.12	−	267...	6.9	+	414...	5.2	−
324...	7.11	+	381...	5.09	−	319...	6.8	−L	438...	5.1	+
267...	7.10	+	344...	5.05	−	366...	6.8	+	436...	5.0	−
310...	7.08	+	335...	5.05	−	376...	6.8	−L	339...	5.0	−
410...	7.06	−L	313...	5.05	−	410...	6.8	−L	359...	5.0	−
369...	7.05	+	431...	5.03	+	440...	6.8	+	362...	5.0	−
402...	7.03	+	353...	5.00	−	369...	6.7	+	373...	5.0	−
311...	7.01	−L	316...	5.00	−	402...	6.7	+	377...	5.0	−
334...	6.96	+	438...	4.98	+	432...	6.7	+	380...	5.0	−
322...	6.93	−L	436...	4.98	−	269...	6.7	+	382...	5.0	−
376...	6.86	−L	377...	4.98	−	412...	6.7	−L	404...	5.0	−
434...	6.84	+	414...	4.97	−	311...	6.6	−L	427...	5.0	−
330...	6.84	+	266...	4.97	−	349...	6.6	+	320...	4.9	−
331...	6.79	+	446...	4.97	−	360...	6.6	−L	323...	4.9	−
317...	6.78	−L	327...	4.93	+	384...	6.6	−L	325...	4.9	−
433...	6.78	−L	380...	4.93	−	391...	6.6	−L	327...	4.9	+
398...	6.77	−L	359...	4.93	−	398...	6.6	−L	335...	4.9	−
349...	6.76	+	441...	4.90	−	433...	6.6	−L	397...	4.9	−
437...	6.72	+	427...	4.90	−	332...	6.5	−L	405...	4.9	−
319...	6.71	−L	323...	4.90	−	374...	6.5	−L	407...	4.9	−
360...	6.69	−L	378...	4.90	−	383...	6.5	−L	415...	4.9	−
302...	6.64	+	390...	4.90	−	399...	6.5	−L	418...	4.9	+
412...	6.62	−L	326...	4.83	−	317...	6.4	−L	441...	4.9	−
321...	6.52	−L	406...	4.83	−	321...	6.4	−L	442...	4.9	−
354...	6.51	+	407...	4.82	−	304...	6.3	+	313...	4.8	−
374...	6.44	−L	411...	4.82	−	308...	6.3	−	326...	4.8	−
383...	6.29	−L	415...	4.81	−	310...	6.3	+	344...	4.8	−
413...	6.28	−	320...	4.80	−	352...	6.3	+	353...	4.8	−
384...	6.27	−L	325...	4.78	−	437...	6.3	+	364...	4.8	−
435...	6.23	−L	364...	4.76	−	347...	6.2	+	371...	4.8	−
332...	6.23	−L	405...	4.75	−	330...	6.2	+	375...	4.8	−
350...	6.20	+	404...	4.70	−	365...	6.2	−L	378...	4.8	−
394...	6.18	−L	389...	4.70	−	367...	6.2	−L	446...	4.8	−
367...	6.18	−L	371...	4.70	−	408...	6.2	−L	430...	4.8	−
347...	6.17	+	356...	4.70	−	434...	6.2	+	425...	4.8	−
401...	6.15	+	418...	4.68	+	306...	6.1	+	422...	4.8	+
408...	6.12	−L	425...	4.66	−	363...	6.1	−	420...	4.8	−
416...	6.07	−L	417...	4.66	−	394...	6.0	−L	417...	4.8	−
315...	6.07	−	382...	4.63	−	413...	6.0	−	411...	4.8	−
361...	6.05	−L	420...	4.59	−	301...	5.9	−	390...	4.8	−
314...	6.03	−	395...	4.58	−	305...	5.9	−	389...	4.8	−
318...	6.01	−L	430...	4.58	−	307...	5.9	−	356...	4.7	−
340...	5.93	−	422...	4.53	+	314...	5.9	−	392...	4.7	−
328...	5.88	−	393...	4.51	−	315...	5.9	−	393...	4.7	−
365...	5.88	−	392...	4.48	−	318...	5.9	−	395...	4.7	−
399...	5.88	−	342...	4.44	−	343...	5.9	−	265...	4.7	−
363...	5.85	−	265...	4.36	−	345...	5.9	−	316...	4.6	−
343...	5.83	−	400...	4.31	−	346...	5.9	−	342...	4.6	−
308...	5.81	−	449...	4.24	−	416...	5.9	−	348...	4.6	−
301...	5.76	−	373...	4.22	−	354...	5.8	+	355...	4.6	−
357...	5.73	−	348...	4.21	−	361...	5.8	−	388...	4.6	−
304...	5.73	+	355...	4.19	−	409...	5.8	−	400...	4.6	−
305...	5.69	−	387...	4.10	−	435...	5.7	−	424...	4.6	−
419...	5.69	+	424...	4.09	−	385...	5.7	−	426...	4.6	−
423...	5.68	+	426...	4.02	−	372...	5.7	−	428...	4.6	−
341...	5.66	−	388...	4.01	−	302...	5.6	+	449...	4.6	−
345...	5.66	−	428...	3.99	−	340...	5.6	−	379...	4.4	−
396...	5.64	−	358...	3.80	−	386...	5.6	−	387...	4.4	−
306...	5.61	+	379...	3.73	−	396...	5.6	−	358...	4.3	−

It could not be expected that the mere correcting of the reaction of an acid, Azotobacter-free soil would bring about the establishment of an Azotobacter flora, unless accompanied by natural or artificial inoculation. Artificial inoculation with Azotobacter has been practiced to a very limited extent and it is probable that none of the soils examined have received such treatment. Natural inoculation would probably eventually occur in all cases. The rapidity with which it would take place would depend primarily upon the proximity of soils containing Azotobacter and upon the activity of the agencies operating in the transfer of soil from one to the other. Among these agencies would be wind, drainage, animals, insects, birds, etc., and the more or less artificial transfer upon agricultural tools and machinery. If soils containing Azotobacter are in close proximity, and the means of transfer are active, inoculation may occur very rapidly. For, in an effort to study the effect of inoculation upon adjacent plots, the writer found it impossible to prevent inoculation under certain conditions, while under other conditions such inoculation has not appeared during a period of three years. Some of the limed Azotobacter-free soils here recorded have been receiving lime for many years. As previously mentioned, many of the plots to which lime has been applied are located on rather large areas of strongly acid soils. Such plots may be some distance from areas that contain Azotobacter and, hence, chances for natural inoculation are poor. On the other hand, the writer repeatedly cultured the fertility plots of the Missouri Agricultural Experiment Station for Azotobacter during the years 1912–1914 with negative results. During 1914–15 certain of these plots received an addition of lime. When cultured for Azotobacter again in 1919 a vigorous flora was found to be present. At this last examination the reaction of the soil from a plot that had been limed was found to be neutral (soil No. 267, Table XI). Soil from adjacent unlimed plots was found to be strongly acid (soils Nos. 265 and 266, Table XI). In other words, decreasing the hydrogen-ion concentration of this soil to a point below 1×10^{-6} has resulted in the establishment of a vigorous Azotobacter flora in a comparatively short time. Other soils not far from these plots have been found to contain Azotobacter and it may be assumed that natural inoculation readily took place. It is not uncommon to find adjacent plots or areas of natural soil one of which contains Azotobacter while the other does not.

Summary of Tables XI and XII

Number of soils examined	154
Number of soils containing Azotobacter	42
Number of soils not containing Azotobacter	112
Average mgm. nitrogen fixed, 148 soils	5.30
Average mgm. nitrogen fixed, 40 soils containing Azotobacter	7.35
Average mgm. nitrogen fixed, 108 soils not containing Azotobacter	4.53
Number of soils electrometric P_H 6.0 or above	60
Number of soils electrometric P_H below 6.0	94
Number of soils colorimetric P_H 6.0 or above	57
Number of soils colorimetric P_H below 6.0	97
Number of soils electrometric P_H 6.0 or above containing Azotobacter	32
Number of soils electrometric P_H 6.0 or above not containing Azotobacter	28
Number of limed soils electrometric P_H 6.0 or above not containing Azotobacter	24
Number of normal soils electrometric P_H 6.0 or above not containing Azotobacter	4
Number of soils electrometric P_H below 6.0 containing Azotobacter	10
Number of soils electrometric P_H below 6.0 not containing Azotobacter	84

Number of soils colorimetric P_H 6.0 or above containing Azotobacter	31
Number of soils colorimetric P_H 6.0 or above not containing Azotobacter	26
Number of limed soils colorimetric P_H 6.0 or above not containing Azotobacter	22
Number of normal soils colorimetric P_H 6.0 or above not containing Azotobacter	4
Number of soils colorimetric P_H below 6.0 containing Azotobacter	11
Number of soils colorimetric P_H below 6.0 not containing Azotobacter	86
Average electrometric P_H, 154 soils	5.72
Average colorimetric P_H, 154 soils	5.70
Average electrometric P_H, 42 soils containing Azotobacter	6.70
Average electrometric P_H, 112 soils not containing Azotobacter	5.35
Average colorimetric P_H, 42 soils containing Azotobacter	6.46
Average colorimetric P_H, 112 soils not containing Azotobacter	5.42
Association coefficient based on electrometric P_H determinations	0.971
Association coefficient based on colorimetric P_H determinations	0.968

Liming is an artificial method by which the reaction of soils can suddenly be changed from an unfavorable to a favorable condition for the growth of Azotobacter. Unless accompanied by inoculation with Azotobacter, soils that have thus been rendered favorable and do not contain Azotobacter should not be classed with soils that normally have a favorable reaction and do not contain Azotobacter. For these reasons the limed soils with reactions of P_H 6.0 or above and not containing Azotobacter have been separated from the others and are not considered in the association coefficient caluclations.

In Series IV there were 28 soils tested by the electrometric and 26 by the colorimetric method (32 different soils) with a reaction of P_H 6.0 or above that did not contain Azotobacter. Twenty-four of the former and 22 of the latter are known to have been limed more or less recently. There were, therefore, only 6 unlimed soils with P_H 6.0 or above by one of the methods that did not contain Azotobacter. Five of the 6 reacted more acid than P_H 6.0 by one of the methods, leaving only 1 unlimed soil definitely more acid than P_H 6.0 and not containing Azotobacter. It is probable that lime has been applied to some of the soils of which no record is available. In a few instances the data indicate that some unknown factor has been influencing the reaction. (Compare soils 413 with 414, 438 with 439, 399 with 400, and 363 with 364.) It is believed that the data on history were incorrectly recorded in a few instances. There were 10 soils that reacted electrometrically and 11 colorimetrically (13 different soils) more acid than P_H 6.0 in which no Azotobacter were recorded. Five of these were less acid than P_H 6.0 by one of the methods; not one of the remaining 8 had a typical Azotobacter growth accompanied by good fixation of nitrogen in both samples.

GENERAL SUMMARY AND DISCUSSION

Number of soils examined	418
Number of soils containing Azotobacter	199
Number of soils not containing Azotobacter	219
Average mgm. nitrogen fixed, 367 soils	6.36
Average mgm. nitrogen fixed, 174 soils containing Azotobacter	8.30
Average mgm. nitrogen fixed, 193 soils not containing Azotobacter	4.61
Number of soils electrometric P_H 6.0 or above	207
Number of soils electrometric P_H below 6.0	211
Number of soils colorimetric P_H 6.0 or above	206
Number of soils colorimetric P_H below 6.0	212
Number of soils electrometric P_H 6.0 or above containing Azotobacter	165
Number of soils electrometric P_H 6.0 or above not containing Azotobacter	42
Number of limed soils electrometric P_H 6.0 or above not containing Azotobacter	24

Number of normal soils electrometric P_H 6.0 or above not containing Azotobacter	18
Number of soils electrometric P_H below 6.0 containing Azotobacter	34
Number of soils electrometric P_H below 6.0 not containing Azotobacter	177
Number of soils colorimetric P_H 6.0 or above containing Azotobacter	166
Number of soils colorimetric P_H 6.0 or above not containing Azotobacter	40
Number of limed soils colorimetric P_H 6.0 or above not containing Azotobacter	22
Number of normal soils colorimetric P_H 6.0 or above not containing Azotobacter	18
Number of soils colorimetric P_H below 6.0 containing Azotobacter	33
Number of soils colorimetric P_H below 6.0 not containing Azotobacter	179
Average electrometric P_H, 398 soils	6.09
Average colorimetric P_H, 418 soils	6.11
Average electrometric P_H, 193 soils containing Azotobacter	6.88
Average electrometric P_H, 205 soils not containing Azotobacter	5.44
Average colorimetric P_H, 199 soils containing Azotobacter	6.72
Average colorimetric P_H, 219 soils not containing Azotobacter	5.56
Association coefficient based on electrometric P_H determinations	0.959
Association coefficient based on colorimetric P_H determinations	0.961

In this summary there are a few points to which the writer wishes to call special attention. Little is known of the practical significance of Azotobacter in the nitrogen economy of soils. After carefully reviewing all the available literature and data on the subject Greaves (*8*) says:

> In conclusion, it may be stated that, although the part played by Azotobacter in maintaining the nitrogen of the soil has not been definitely measured, it is nevertheless an important factor. It is, therefore, conservative to state that these organisms under favorable conditions add from 15 to 40 pounds of available nitrogen to each acre of soil yearly.

If these figures are a conservative estimate of the quantity of nitrogen fixed by these organisms, the presence or absence of such a flora in soils is of enormous economic importance. Yet, more than half the soils examined during the past few years apparently do not contain Azotobacter. There are organisms present in practically all soils capable of fixing some nitrogen under laboratory conditions. The quantity of nitrogen fixed under the conditions of these experiments is only approximately half as great in the absence of Azotobacter as when they are present. It is believed that under actual soil conditions the differences are much more marked than these figures indicate. If this important group of organisms is absent from such a large per cent of soils, what are the factors controlling their presence and absence and how can the unfavorable conditions be remedied?

In a former publication (*2*) an apparent close correlation between the absolute reaction of the soil solution and the presence of Azotobacter in soils was shown. The present data demonstrate the existence of such a correlation. By using Yule's association formula the very high coefficient of 0.96 is obtained, indicating an unmistakable correlation. Very few soils in which the hydrogen-ion concentrations exceed 1×10^{-6} contain Azotobacter, while practically ll soils with a hydrogen-ion concentration less than 1×10^{-6} contain an active Azotobacter flora. The per cent of soils of different hydrogen-ion concentrations containing Azotobacter is found in Table XIV and shown graphically in figure 1.

TABLE XIII.—*Comparison of the presence of Azotobacter, nitrogen fixed, and reaction of same soils collected 2 years apart*

Soil No.		Azotobacter.		Nitrogen fixed.		Colorimetric P_H.		Electrometric P_H.	
1918	1920	1918	1920	1918	1920	1918	1920	1918	1920
				Mgm.	*Mgm.*				
1	101	+	+	10.3	10.7	6.9	6.7	.....	6.71
2	102	−	−	2.0	6.3	5.4	5.0	.....	3.78
3	103	−	−	3.0	5.4	5.6	5.4	.....	5.27
4	104	+	+	6.8	10.5	6.9	7.1	.....	.....
5	105	+	+	5.4	11.8	7.1	7.2	.....	7.08
6	106	−	−	4.4	6.1	5.7	5.7	.....	5.25
15	133	+	+	10.3	13.5	7.4	7.3	.....	7.45
16	136	−	+?	4.0	10.5	5.6	5.4	.....	4.88
17	137	−	−	3.0	4.3	5.5	5.8	.....	5.58
18	138	−	−	3.7	3.0	5.6	5.4	.....	5.05
31	107	+	+	10.6	10.2	7.5	7.4	7.71	7.70
32	108	−	+?	5.6	8.5	5.9	5.6	5.85	5.48
33	109	−	+	4.0	9.5	6.7	6.6	6.56	6.47
34	110	−	+	4.5	7.9	6.8	7.4	6.74	7.61
35	111	+	+	6.8	9.1	7.6	6.6	7.62	6.39
36	113	+	+	9.2	8.9	6.0	5.6	6.12	5.85
37	114	−	+	5.4	8.9	6.2	5.8	6.07	5.59
38	115	−	+	3.9	8.6	5.6	5.4	5.59	5.51
39	116	+	+	8.6	10.0	6.1	6.0	6.05	5.90
40	119	+	+	8.1	9.8	7.0	7.1	6.72	7.00
49	126	−	−	2.7	8.0	5.5	5.5	5.03	5.19
50	127	−	−	4.0	6.6	5.8	5.5	5.32	5.10
51	128	−	−	3.2	5.1	5.3	5.9	5.41	5.78
52	129	+	+	5.5	10.8	7.3	7.6	7.27	7.30
53	130	+	+	9.0	7.3	7.7	7.4	7.79	7.52
54	131	+	−	7.4	7.7	6.0	5.8	6.08	5.47
61	120	−	+	3.7	6.0	5.5	5.8	5.51	5.68
63	124	+	+	11.0	9.0	7.4	6.9	7.43	7.06
64	122	+	+?	8.3	8.6	6.1	5.8	6.35	5.51
65	123	−	+	5.2	8.8	5.7	5.9	5.94	5.64
66	121	−	−	3.8	7.6	5.7	5.7	5.81	5.24
77	134	−	−	4.2	7.2	5.6	5.4	5.34	4.88
79	143	+	+	7.6	7.0	7.7	8.1	8.48	8.77
80	144	+	+	9.2	8.8	7.6	7.3	7.84	7.72
82	142	+	+	8.2	9.7	7.7	7.8	7.60	7.59
83	145	+	+	9.9	7.5	7.6	7.2	7.72	7.94

TABLE XIV.—*The number and percentage of soils of various reactions containing Azotobacter*

P_H reaction.	Electrometric.					Colorimetric.				
	Number of soils.	Azotobacter.				Number of soils.	Azotobacter.			
		Present.		Absent.			Present.		Absent.	
		No.	*Per cent.*	*No.*	*Per cent.*		*No.*	*Per cent.*	*No.*	*Per cent.*
Above 7.50	57	57	100	0	0	32	32	100	0	0
7.00 to 7.49	49	45	92	4	8	60	58	97	2	3
6.50 to 6.99	33	27	82	6	18	45	39	90	6	10
6.00 to 6.49	38	30	80	8	20	47	37	80	10	20
5.50 to 5.99	64	22	34	42	66	122	24	20	98	80
5.00 to 5.49	75	7	9	68	91	43	6	14	37	86
4.50 to 4.99	42	5	12	37	88	44	3	7	41	93
Below 4.50	16	0	0	16	100	3	0	0	3	100
Total	374	193	...	181	...	396	199	...	197	...

The existence in soils of an excess of hydrogen over hydroxyl-ions is no longer questioned. In 75 per cent of the 418 soils tested an excess of hydrogen over hydroxyl-ions was found whether the concentration was measured electrometrically or colorimetrically. A large number of examples are on record showing the toxic effect of a high concentration of hydrogen-ions upon bacteria. The writer has recently shown (*6*) that pure cultures of Azotobacter will not grow in laboratory media with a hydrogen-ion concentration appreciably in excess of 1×10^{-6}.

It is quite generally accepted that soil acidity is indirectly responsible for certain plant root diseases, and it is possible that the influence of the absolute reaction of the soil solution upon Azotobacter here indicated is also indirect. However, since the same identical limiting hydrogen-ion concentration has been found with pure cultures, in laboratory media (*6*) as in soil solutions, it is believed that the recorded absence of this group

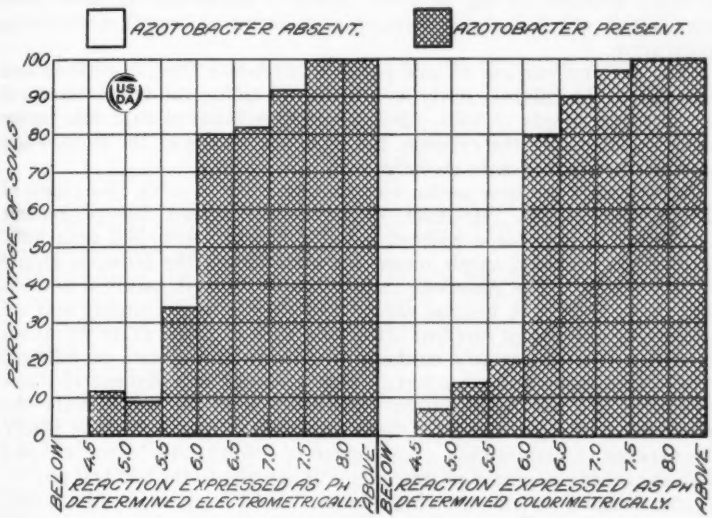

FIG. 1.—Percentage of soils of different reactions containing Azotobacter

of organisms in the soils examined is in most instances correct and is directly due to the toxic effect of the high concentration of hydrogen-ions existing in the soil solution. Furthermore, it is believed that the maximum hydrogen-ion concentration tolerated by this group of organisms is very near 1×10^{-6} in soils as well as in laboratory media. Whether or not the hydrogen-ions are the ultimate limiting agent, the fact has been clearly established that the two are definitely associated. It is also evident from the data accumulated in this laboratory and shown elsewhere (*3*) that the same factors that will raise the hydrogen-ion concentration of a soil solution appreciably above, or lower it below, 1×10^{-6} will also render the soil capable or incapable, as the case may be, of supporting an Azotobacter flora.

How can the unfavorable conditions for the existence and growth of Azotobacter in soils be corrected? Attention has been called to the fact that a reduction in the hydrogen-ion concentration is probably the

one essential condition to be fulfilled, in rendering a soil capable of supporting Azotobacter. This has been accomplished experimentally in a number of different ways. Perhaps the simplest and most economical one is the addition of sufficient lime to reduce the hydrogen-ion concentration to a point below 1×10^{-6}. Attention has also been called (*3*) to the ease with which this can be done in the laboratory. In a forthcoming publication it will be shown that the same is true under natural soil conditions. The most acid soil that has been encountered locally required approximately 18,000 pounds of calcium carbonate to reduce the acidity to below P_H 6.0. Three years ago sufficient lime to accomplish this was added under natural conditions accompanied by inoculation both with soil containing Azotobacter and with cultures grown in the laboratory. These plots still contain a vigorous Azotobacter flora and will fix, in laboratory media, two to three times as much nitrogen as will adjacent, similarly inoculated, unlimed plots from which the Azotobacter disappeared within a few days following inocu'ation.

It is not the purpose of this paper to advocate the use of lime and Azotobacter inoculation solely as a means of aiding the maintenance of the nitrogen supply of soils. For, while it is believed that this factor alone would justify the expense, the economic phases of the proposition have not been sufficiently investigated.

Experiments are now under way that promise to solve this phase of the problem locally. However, it is generally agreed that permanent agriculture is impossible without a soil sufficiently supplied with lime. Nature has provided ample means for maintaining the nitrogen supply of native soils. The American farmer has so ignored nature's methods that many soils have become depleted of their nitrogen supply and are productive only when supplied artificially with nitrogen at an enormous expense. One of nature's methods is through the agency of different types of nitrogen-fixing bacteria. One essential requirement of these organisms is a low hydrogen-ion concentration in the soil. While the quantity of nitrogen fixed by these organisms might not alone justify the expense of maintaining a low degree of acidity, this factor, coupled with the other recognized physicochemical benefits to the soil and physiological benefits to the growing plants, would unquestionably justify such a practice. The possible influence of adequate liming upon the nitrogen economy of the soil is mentioned as an added stimulus to the use of lime.

Another point to be noted is the general similarity of results, whether based upon the electrometric or the colorimetric method of determining the hydrogen-ion concentration of the soil solution. The individual determinations frequently do not agree as closely as might be desired. Yet the conclusions, so far as this study is concerned, would be identical regardless of data upon which they were based. In a general way the farther removed from neutrality, the wider the variation between the two methods. The electrometric method usually recorded a higher or lower hydrogen-ion concentration, as the case might be, than the colorimetric method if the soil were appreciably acid or alkaline. It is believed, however, that the general agreement between the two methods is sufficiently close to justify the use of the much quicker and less expensive colorimetric method as an aid in the solution of many soil problems, particularly where the initial expense prohibits the purchase of the necessary expensive electrometric apparatus.

The possibility of a biological means of determining the lime requirements of soils is indicated by the data here presented. If a series of groups or of individual microorganisms with definite limiting hydrogen-ion concentrations could be isolated from soils it would only be necessary to determine the presence or absence of certain species in order to establish the absolute reaction of the soil solution in situ. Furthermore, it would be comparatively simple to ascertain how much lime to add in order to render the soil capable of supporting the various species. It has been possible to determine with an accuracy of a few hundred pounds the quantity of lime necessary to add to soils more acid than P_H 6.0 in order to render them capable of supporting Azotobacter. It is believed that not until the plant physiologist has established the optimum and limiting hydrogen-ion concentrations of the soil solution for the various agricultural plants, and the bacteriologist has associated these limits with definite groups of microorganisms, will a true and accurate physiological basis for determining and correcting the reaction of soils be possible. Azotobacter can now be used as a biological means of separating soils into two groups, depending upon reaction; and the quantity of lime necessary to change the reaction of the more acid group into the less acid group can be definitely determined.

CONCLUSIONS

(1) Seventy-five per cent of the 418 soils examined were found to be acid. Fifty per cent were more acid than P_H 6.0 and 50 per cent apparently did not contain Azotobacter.

(2) In general the electrometric and colorimetric methods of determining the hydrogen-ion concentration of the soil solution agreed.

(3) A definite and very close correlation has been established between the absolute reaction of the soil solution and the presence or absence of Azotobacter in the soil.

(4) Very few soils more acid than indicated by a hydrogen-ion concentration of 1×10^{-6} contain Azotobacter, while this group of organisms is in most instances present in soils with a lower hydrogen-ion concentration. Since a very similar limiting hydrogen-ion concentration has been found for pure cultures of Azotobacter in laboratory media, it is believed that the very close correlation existing between the reaction and the presence of Azotobacter indicates that the absolute reaction is of paramount importance, if not the actual limiting factor, in controlling the presence of this group of organisms in soils.

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A STUDY OF FACTORS AFFECTING THE NITROGEN CONTENT OF WHEAT AND OF THE CHANGES THAT OCCUR DURING THE DEVELOPMENT OF WHEAT[1]

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INTRODUCTION

The subject matter of this paper pertains to a study of the nitrogen content of wheat produced under various experimental conditions and a study of changes that occur during the development of wheat. The investigation had its inception in studies of nitrogen content of wheat by Thatcher (*31*, *32*, *33*).[3]

In the States of California, parts of Idaho, Montana, Oregon, Utah, and Washington, wheat, irrespective of variety, generally develops large, plump kernels of comparatively soft texture. The nitrogen or gluten content of these soft wheats is variable, with localized districts apparently more favorable to the production of wheat with high percentages of nitrogen or gluten than is observed in others. Farther east, in the States of North Dakota, South Dakota, Nebraska, Kansas, Minnesota, Missouri, and Iowa the wheat kernels, generally, are of smaller size. These smaller-sized kernels, however, may be plump yet the texture of the grain may be comparatively harder. The percentage of nitrogen or gluten in this harder type of wheat varies but, generally speaking, is comparatively higher than in the softer wheats.

The influence of exchange of seed, breeding new varieties, selections for nitrogen content, soil types, soil treatment, fertilizers, water, and temperature on the percentage of nitrogen in wheat have been studied by various investigators. From the results of investigation, it is held that the variation in composition of wheat is due, chiefly, to variation in environmental conditions, such as rain, sunshine, humidity of the atmosphere, methods of harvesting, methods of cultivations, and amount of manure. The effect of continuous cropping, rotation, the method and time of sowing seed, including the quantity and quality of the seed sown, have also been studied. With the exception of irrigation, the first three of these conditions are wholly uncontrollable; the remainder of them can be regulated, and it is to these that the investigational work has been and should be directed to determine whether or not the nitrogen content of wheat can be increased.

HISTORICAL REVIEW

EFFECT OF CLIMATE ON NITROGEN CONTENT OF WHEAT

Over 60 years ago, Lawes and Gilbert (*7*) concluded from their experiments that long periods of growth produced plump kernels of low nitrogen content. Later these men (*8*) stated that—

> a season favorable for long and continuous growth after heading, produced well developed kernels and larger yields; the mature grain developed under the most favorable conditions contained a high amount of starch and a low amount of potash, phosphoric acid, and nitrogen.

[1] Accepted for publication Oct. 17, 1921.

[2] The author desires to thank Alexander Carlyle, L. F. Seneco, and Sam Bockman for their assistance in looking after the field work.

[3] Reference is made by number (italic) to "Literature used," p. 951-953.

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Richardson (*18*) and Brewer (*3*) concurred that climate exerted the greatest influence. According to Wiley (*39, p. 244; 40*) a cool climate and short periods of growth are favorable for high protein, while a warm climate and long periods of growth are favorable for low protein content. Jensen (*6*) expressed the opinion that a "continental climate" was favorable to the formation of a small grain of high nitrogen content. Schindler (*19*) concluded that a warm, moist climate prolonged the kernel development, resulting in a high starch and low protein wheat. Melikov (*13*) gave heavy rainfall as the cause for low nitrogen content in wheat. LeClerc (*9*) deduced that the differences in composition for any one locality from season to season was due to varying weather conditions. The differences in composition of wheat analyzed by him for different localities were pronounced. Williams (*41*) stated that variation in protein was independent of variety and of soil treatment. He ascribed it to seasonal influences. When the wheat was small or shrunken the protein content was high and vice versa. Thatcher, in a study correlating the protein content of wheat with the rainfall in various districts from which samples were taken, observed that the protein content varied inversely with the total rainfall (*35*). According to Deherain (*5*), slow ripening was favorable to a heavy yield of wheat with average gluten content, while hot summer conditions were favorable to rapid ripening and high gluten content.

LeClerc (*9, p. 202*), in making a comparative study of the composition of wheat grown under arid and humid conditions, concluded from his experiments that the nitrogen content is higher in the wheat grown on the arid and semiarid land than it is found in wheat grown on humid and irrigated land. In other words—

> an excessive amount of rainfall or irrigation is always accompanied by a crop containing a very low percentage of protein.

IRRIGATION WATER

Investigations pertaining to the influence of water on composition of grain have been carried on over a comparatively long period. The irrigation investigations in charge of the Utah Agricultural Experiment Station are especially valuable, and show how the nitrogen content of the grain is increased through the judicious use of water. The report of a progressive study of the nitrogen content of grain by Widstoe (*37*) in 1921 shows that large applications of water may increase slightly the percentage of protein in wheat. The percentage of nitrogen in spring wheat decreases with the increased use of water, ranging from 15.26 per cent protein for 20 inches to 26.7 per cent protein for 4.63 inches. With applications of water ranging from 8.89 inches to 40 inches, the variation in nitrogen content is found to be irregular, but the 40-inch application of water shows higher protein than is found in the wheat receiving either 21 or 30 inches of water.

The time of applying the water appears to be very important, since Widstoe and Stewart (*38*) find that the protein content of wheat decreased from 18.05 per cent to 15.98 with the increased use of water after the middle of July. When water is applied before the first of July, the protein content of the wheat is increased. Stewart and Greaves (*28*) concur with LeClerc (*9, p. 202*) that wheat grown on arid nonirrigated land contains more protein than is found in wheat grown on adjoining irrigated land. Prianischnikov (*17*) finds that high per-

centages of water in the soil lowers the nitrogen content of the grain. He also finds that the growing period for the wheat, undoubtedly due to a weaker soil solution and especially the concentration of nitrates which are inadequate for the best development, is shortened with the use of an increased water supply. Von Seelhorst and Krzymowski (*21*) also find that the ripening period may be shortened by keeping the water content of the soil up to 85 per cent of its capillary capacity.

Preul (*16*) studied the influence of variable amounts of water on the composition of wheat grown on soil rich or poor in fertility. He found that the nitrogen content of the wheat was high when the application of water was kept constantly low. A lack of water during the late stages of growth caused a comparatively low nitrogen content in the grain.

Shutt (*24*, *25*) states that the wheat grown on newly cleared scrub land compared with wheat grown on fallow or cultivated land is physically softer, "piebald," more starchy, and lower in nitrogen content. This finding, he believed is due to the larger amount of water contained in the newly cleared scrub land, causing a prolongation of the growing period of the plant.

SOIL AND FERTILIZERS

The soil as a factor in influencing the composition of wheat has been studied. Thatcher (*32*), LeClerc (*11*), and Shaw and Walters (*22*) are of the opinion that the composition of soil has very little, if any, influence on the composition of grain. Shaw and Walters state that the soil nitrogen content has little, if any, direct influence upon the nitrogen content of grain and that the factor of climate is sufficient to entirely overshadow the soil factor. In connection with these experiments, the work of other investigators on the influence of fertilizers should not be overlooked. Snyder (*26*) states that—

> increasing the amount of nitrogen in the soil, increases the amount of nitrogen in the grain.

Ames (*1*) shows that, without exception, application of nitrogen fertilizer increased the nitrogen content of the grain. The proportion of phosphorus, potassium, and nitrogen in the wheat plant is increased by the addition of these elements to the soil. Whitson, Wells, and Vivian (*36*) believe that under the same seasonal conditions the most important factor in causing variation in the composition of crops is the amount of nitrates in the soil. On the other hand, Soule and Van Atter (*27*) state that the use of fertilizers on the growing of wheat in a rich soil did not appreciably increase the protein content.

INVESTIGATIONAL WORK

I. FACTORS AFFECTING THE NITROGEN CONTENT OF WHEAT

EFFECT OF WIDTH OF ROWS ON NITROGEN CONTENT OF WHEAT

In the earlier investigational work undertaken at this experiment station on the influence of length of growing season, soil, or climatic condition on the percentage of nitrogen in wheat, methods of preparing the soil and seeding were followed which were not considered a part of the investigation but which undoubtedly played a very important part in the results obtained.

It was found that the percentage of nitrogen in the fall-sown wheats was only slightly lower than that in the spring-grown wheat. These similarities in nitrogen content have indicated that there are some factors, beside the length of the growing season, which have influenced the formation of wheat of high nitrogen content. It was further found that wheat grown in the nursery plots contained more nitrogen than was found in wheat grown in variety-test plots or under practical farming conditions. The wheat grown in the nursery contained 18 per cent more nitrogen than was found in the wheat grown in the variety-test plots, and this nitrogen content was considerably higher than was found in the wheat grown under field conditions. These outstanding differences in the percentages of nitrogen pointed to the influence of the method of preparing the soil on the composition of the wheat. It was observed that the wheat grown in the nursery plots was sown in rows 18 inches apart, while the wheat grown in the variety-test plots was seeded 6 inches apart. Furthermore, the nursery plots received a very thorough cultivation, a practice which was not followed in the variety-test plots. These differences in soil treatment and seeding undoubtedly played a very important part in the development of wheat containing different amounts of nitrogen and also indicated the possible reason for the similarity of the percentages of nitrogen in the fall-sown and spring-sown wheats.

The study of the influence of cultivation on wheat sown in rows different distances apart was, therefore, undertaken. The wheat was sown in rows 6, 9, 12, 15, and 18 inches apart. That sown 6 and 9 inches apart could not be properly cultivated but represented farm methods on well-prepared soil. A sample of the same wheat grown on another plot was also analyzed for the purpose of making a comparison of the nitrogen content of the wheat grown in the nursery with that under field conditions. The results are given in Table I.

TABLE I.—*Percentage of nitrogen in wheat (hybrid 143) sown in rows various distances apart and cultivated*

Treatment.	Fall wheat.		Spring wheat.	
	Nitrogen.	Increase over field.	Nitrogen.	Increase over field.[a]
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*
Field grown	2.11			
Nursery grown:				
6 inches apart, end rows	2.30	9.0	2.50	18.5
6 inches apart, middle rows	2.35	11.4	2.56	21.3
9 inches apart, end rows	2.38	12.8	2.46	16.6
9 inches apart, middle rows	2.32	10.0	2.53	19.9
12 inches apart, end rows	2.54	20.4	2.58	22.2
12 inches apart, middle rows	2.48	17.5	2.68	27.0
15 inches apart, end rows	2.53	19.9	2.55	20.8
15 inches apart, middle rows	2.47	17.1	2.56	24.6
18 inches apart, end rows	2.66	26.1	2.56	21.3
18 inches apart, middle rows	2.66	26.1	2.77	31.2

[a] Calculated using (2.11 per cent) field sample of fall wheat as a basis.

It will be noted that the smallest increase of nursery-grown over field-grown wheat is 9 per cent, while the largest increase is 31.2 per cent. The fall wheat grown in rows 18 inches apart in the nursery shows 15

per cent increase in nitrogen over that grown in rows 6 inches apart. The same comparison with spring wheat shows a 10.8 per cent increase. The amount of nitrogen in the spring wheat containing the lowest percentage of nitrogen is 6.95 per cent greater than the corresponding fall wheat. Comparing the highest percentages in spring and fall wheats, it is found that the amount in the spring wheat is greater by 4.1 per cent.

From the results in Table I it will be noted that the nitrogen content of the wheat was increased by increasing the distance between the rows. This is more noticeable in the fall than in the spring seeding. Omitting the rows of 6 and 9 inch seeding, the percentage of nitrogen in the fall and spring is only slightly in favor of the latter. Owing to the fact that the extremes of variation in nitrogen are not as marked in the spring as in the fall seeding, it is of interest to note that the fall-sown wheat shows a slightly higher nitrogen content for the wheat grown in the rows next to the end than in the middle rows, with the exception of that grown 18 inches apart, where there is no difference observed. The spring wheat shows higher results for nitrogen in the middle rows than it does for those next the end.

EFFECT OF VARYING AMOUNTS OF WATER AND WIDTH OF ROWS ON NITROGEN CONTENT

In a subsequent experiment, a study of the influence of varying amounts of water on the nitrogen content of wheat was made. The field was divided into two series of plots, and two varieties of wheat were grown on them. The rows were sown 6, 9, 12, 15, and 18 inches apart. The amount of water used in the experiment ranged from 8.78 inches rainfall to 12, 16, and 20 inches. The land was cultivated at various times during the growing period in order to remove weeds and maintain a mulch. The average results for nitrogen content of the wheat are recorded in Table II.

TABLE II.—*Average percentage of nitrogen in wheat grown on cultivated plots receiving varying amounts of water*

Variety.	Distance.	Plot I, 8.78 inches of water.[a]	Plot II, 12 inches of water.	Plot III, 16 inches of water.	Plot IV, 20 inches of water.
	Inches.				
Sonora	6	2.37	2.36	2.23	2.22
	9	2.13	2.13	2.15	2.03
	12	2.01	2.21	2.21	2.17
	15	2.26	2.21	2.12	2.30
	18	2.53	2.46	2.31	2.48
Average		2.26	2.27	2.20	2.24
Hybrid 143	6	2.21	2.13	2.29	2.26
	9	2.03	1.88	1.88	2.12
	12	2.03	2.06	2.04	2.16
	15	2.02	2.39	1.89	2.08
	18	2.17	2.42	2.20	2.30
Average		2.09	2.18	2.06	2.18

[a] Total precipitation at Grandview in 1914 was 8.78 inches.

There are some abnormal results which undoubtedly are due to the washing of the plant food from some of the plants toward others, because the lowest results for nitrogen were obtained where the irrigation water entered the plots while the highest results were found in the adjacent rows.

In general, the composition of the wheat grown on plots receiving 20 inches of total water shows approximately as high nitrogen content as was found on any of the other plots. The spreading of the rows 18 inches apart appears to have influenced the nitrogen content of the wheat.

The following year the experiment was repeated with the exception of distributing the irrigation water, especially for the 16 and 20 inch irrigated plots, over a longer period. The purpose of this change was to prevent an excessive dilution and washing of the plant food. The results for nitrogen are recorded in Table III.

TABLE III.—*Average percentage of nitrogen in wheat grown on cultivated plots receiving varying amounts of water*

Variety.	Distance.	Plot I, rainfall only.[a]	Plot II, 12 inches of water.	Plot III, 16 inches of water.	Plot IV, 20 inches of water.
	Inches.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Sonora	6	1.96	2.29	2.41	2.22
	9	1.91	2.05	2.17	2.10
	12	1.97	2.12	2.08	2.20
	15	2.08	2.10	2.15	2.17
	18	2.44	2.21	2.21	2.29
Average		2.07	2.15	2.20	2.20
Hybrid 143	6	1.83	2.16	2.30	2.21
	9	1.81	1.81	1.93	2.10
	12	1.81	1.97	2.00	2.08
	15	2.01	2.20	1.98	2.06
	18	2.21	2.44	2.14	2.24
Average		1.93	2.12	2.08	2.14

[a] Total precipitation at Grandview in 1914 was 8.78 inches.

The wheat sown in rows 18 inches apart has evidently been benefited, since it contains more nitrogen than was found in wheat sown in rows 6 and 12 inches apart.

II. CHANGES THAT OCCUR DURING THE DEVELOPMENT OF WHEAT

DISTRIBUTION OF NITROGEN IN KERNEL AND PLANT

In order to know more definitely what conditions are beneficial to the production of high percentages of nitrogen in the grain it is of considerable importance to know something with regard to the amount of nitrogen in the whole plant as well as different parts of the plant, especially after the development of the kernel has commenced. For this study, plants of nearly equal lengths and weights were selected at 7-day intervals. Nitrogen determinations were made on the stems and leaves between the joints or nodes, above the top node, and on kernel and chaff. The data in Table IV show the percentages of nitrogen in the plant exclusive

of kernels, percentage of nitrogen in kernels, average weight of kernel, total and average weight of nitrogen in kernel. The data in Table V show the grams of nitrogen in the chaff, above the top nodes, below the top node, below the second node from top, and in some cases (when present) below third node, as well as total weight of nitrogen in the plants.

TABLE IV.—*Percentage distribution of nitrogen in plant and kernels, Hegnauer tract*

Date.	Nitrogen in plant, not kernel.	Nitrogen in kernel.	Average weight of kernel.	Total nitrogen in kernels.	Average nitrogen in kernels.
	Per cent.	*Per cent.*	*Gm.*	*Gm.*	*Gm.*
July 1	0.871	2.23	0.00402	0.00709	0.0000885
8	.800	2.12	.00976	.0155	.000207
15	.571	1.98	.01305	.0155	.000258
22	.529	1.87	.0298	.0484	.000557
29	.460	1.51	.0356	.0334	.000528

TABLE V.—*Weight and distribution of nitrogen in wheat plant, Hegnauer tract*

Date.	Chaff.	Above top node.	Below top node.	Below second node.	Below third node.	Total plant.
	Gm.	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
July 1	0.0074	0.0196	0.00569	0.00540	0.00239	0.04757
8	.0076	.0102	.0092	.0042	.0029	.0496
15	.00401	.0091	.0056	.0042	.0020	.04041
22	.0048	.0089	.0065	.0022	.....	.0408
29	.0022	.0038	.0012	.0014	.....	.0420

It is a well-established fact that the percentage of nitrogen in the plant proper decreases with the continuous growth of the plant. This is clearly seen from the results given in Table IV, which also shows the nitrogen percentage of the kernel decreasing with the progressive development and increased weights of the kernel when grown under field conditions.

The weight distribution of the nitrogen in the several parts of the plant furnishes us some interesting data regarding the changes that take place as the kernels develop in size. It will be seen from the data given in Table V that the nitrogen (reading down the column) moves from the lowest to uppermost part of the plant, and the nitrogen is evidently the first to be drawn upon for the formation of the kernels. At the time of the filling of the kernel the highest percentage of nitrogen is found in the part of the plant above the top node and in the chaff, and the lowest percentage of nitrogen is found in the part below the lowest node. As the nitrogen enters into the kernel, the quantity in the chaff appears to be depleted first, followed by the final draft from the straw above the top node. Therefore, if the conditions are favorable for normal development, all of the excess nitrogen in the different parts of the plant will be moved upward and transferred into the kernel.

EFFECT OF IRRIGATION WATER ON WEIGHT OF KERNEL AND VARIATION OF NITROGEN DURING GROWTH

It is generally believed that the final filling of the kernel takes place some time immediately after the grain has been cut. This belief has been based almost entirely on theory, and there is nothing in literature of a scientific nature that informs us as to its correctness. The importance of water in assisting the transference of the nitrogen into the grain has been studied, so that we are aware of the influence of water in increasing the weight and nitrogen content of the grain in the early stages of kernel development.

The plan for the first year of the experiment was to study the nitrogen composition of the developing grain at intervals of one week. The land was divided into five different plots as follows: Plot I, receiving rainfall amounting to approximately 8.78 inches; plot II, irrigated sufficiently to make the total rain and irrigation water equal to 15 inches; plot III, given 20 inches of water; plot IV, given 25 inches of water; and plot V, given 30 inches of water. Blocks of entire plants were pulled at each period and stored. Afterwards the grain was threshed out and analyzed. The results for average weight of kernels are recorded in Table VI, and average results for percentage of nitrogen are recorded in Table VII.

TABLE VI.—*Weight of grain grown with various amounts of water*

Date.	Plot I, rainfall only.	Plot II, 15 inches of water.	Plot III, 20 inches of water.	Plot IV, 25 inches of water.	Plot V, 30 inches of water.
	Gm.	*Gm.*	*Gm.*	*Gm.*	*Gm.*
July 1	0.0069	0.0052	0.0061	0.0051	0.0064
8	.0151	.0148	.0144	.0136	.0117
15	.0262	.0263	.0262	.0238	.0230
22	.0322	.0335	.0375	.0347	.0346
29	.0333	.0356	.0407	.0379	.0398

TABLE VII.—*Nitrogen in grain grown with various amounts of water*

Date.	Plot I, rainfall only.	Plot II, 15 inches of water.	Plot III, 20 inches of water.	Plot IV, 25 inches of water.	Plot V, 30 inches of water.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
July 1	3.00	3.21	3.04	2.95	2.63
8	2.53	2.38	2.21	1.95	2.60
15	2.42	2.29	1.82	1.92	1.97
22	2.56	2.31	2.07	1.97	2.04
29	2.73	2.20	2.14	1.92	2.02

The highest average weight of the kernels for the first two weeks of development was obtained in the nonirrigated plot I. At the end of the third week the weight of the kernels were equally as high in plots II and III as in plot I. For the fourth week period and at maturity, the weight of the kernels in plots II, III, IV, and V exceeded those of plot I.

With reference to nitrogen content for the first week, plots I, II, and IV have about the same values. Wheat from plot II tested the highest and wheat from plot V analyzed the lowest for nitrogen content. In the

second week the results for nitrogen were in favor of wheat grown on plot II with plot I wheat showing nearly as much nitrogen. At the third, fourth, and maturity periods the highest nitrogen content was found in plot I wheat. Wheat from plots II, III, and IV did not change appreciably after the drop in nitrogen from the first to the second week. With the exception of the wheat grown on nonirrigated land, it should be noted that the varying amounts of water applied had not appreciably affected the nitrogen content of the mature grain.

A subsequent trial was made with two varieties of wheat for the purpose of studying the influence of varying amounts of water when distributed over a longer period of growth than in the previous experiment. The average weights of kernels in the Sonora and Hybrid 143 wheats are recorded in Table VIII.

TABLE VIII.—*Weight of kernels as influenced by applying varying amounts of water over a wide period and then cultivating after water application*

Variety.	Distance.	Plot I, rainfall only.	Plot II, 12 inches of water.	Plot III, 16 inches of water.	Plot IV, 20 inches of water.
	Inches.	*Gm.*	*Gm.*	*Gm.*	*Gm.*
Sonora	6	0.0506	0.0503	0.0503	0.0506
	9	.0499	.0516	.0507	.0520
	12	.0507	.0515	.0507	.0517
	15	.0505	.0514	.0520	.0505
	18	.0515	.0524	.0523	.0503
Hybrid 143	6	.0332	.0331	.0344	.0338
	9	.0329	.0326	.0335	.0327
	12	.0332	.0334	.0336	.0344
	15	.0327	.0330	.0349	.0346
	18	.0343	.0346	.0350	.0350

Apparently, the variation in amount of water used or the distance of the rows has not had any effect on the Sonora wheat, since the weights of the kernels are fairly uniform, regardless of conditions of the experiments. Hybrid No. 142 wheat increased slightly in weight in the 15 and 18 inch rows, where 20 inches of water were applied.

EFFECT OF WATER ON TRANSLOCATION OF NITROGEN

For the purpose of learning whether or not the placing of the wheat plant in water would affect the nitrogen content of the wheat, uniform wheat plants were selected and brought to the laboratory. One-half of the wheat heads and the grain were removed from three-fourths of the samples, and each plant and the grain removed were given corresponding numbers. The heads in the other one-fourth of the samples were not touched. The plants were divided into two lots, one-half of which were placed upright in beakers of water and the other one-half were left standing near but were not placed in water. Following the termination of the experiment, determinations of the weight and the nitrogen content of the grain in both samples were made. The results are recorded in Table IX.

TABLE IX.—*Influence of water in moving nitrogenous and nonnitrogenous material from the stems into the wheat kernel*

	Average weight per kernel.	Average percentage of nitrogen.	Average weight of nitrogen per kernel.
Out of water:	*Gm.*		*Gm.*
Whole heads	0.0071	2.62	0.000186
Half heads	.0077	2.38	.000183
In water:			
Whole heads	.012	3.24	.000389
Half heads	.015	3.23	.000487

The average weight of the kernels at the beginning of the experiment was between 0.0071 and 0.0077 gm. and the nitrogen content varied from 2.38 to 2.62 per cent. At the close of the experiment, whereas the percentages of nitrogen were higher in the whole heads than in the kernels of the half heads, the total weight of nitrogen in both instances was practically the same. The weights of the kernels and percentage of nitrogen in the kernels of the plants which were placed in water had increased. The increase in the weight of kernels and the weight of nitrogen in the one-half of the kernels left on the head was found to be proportionally greater than was obtained for the average of all the kernels left intact or, as it is termed in Table IX, the whole heads. In other words, the increased amount of material in kernels left on one-half the head if distributed to double the number of kernels would have given results similar to those found in all the kernels left intact on the head. Translocation of plant food material has been brought about, and, because of its more highly nitrogeneous character, it is believed that larger quantities of water are required to move the nitrogenous matter than are required to move the nonnitrogenous matter into the kernels. Accordingly, water should prove beneficial to high rather than low nitrogen content. The protein of wheat depends first upon the supply of available nitrogen (*4, v. 10; 5*) for the plant, and second upon movement of the nitrogenous material into the grain.

RELATION OF PERCENTAGE OF MOISTURE IN THE KERNELS TO FILLING

In addition to investigational work mentioned above, the weight of the grain, moisture, starch content, etc., were determined at the various stages of the development of the kernels. The samples were collected every other day, and observations for maximum and minimum temperature and relative humidity were recorded. The results for weight and moisture content of the kernels have been plotted in figure 1.

Apparently no filling occurs in grain having a moisture content of 40 per cent or less. The vertical parts of the curves represent measurements made during periods of desiccation.

The figures for maximum and minimum temperatures and relative humidity have not been submitted because it was impossible to show conclusive relationship between these figures and those for changes of weight of kernels during the period of development. There were periods in the development, however, where the cooler nights were favorable for greater increases in weight than was found to be the case where the warmer nights prevailed. Considering the changes in weight

of the kernel, it should be noted that there were times when very little increase in weight was obtained while at other periods considerable gain was observed. On the whole, it should be noted that the kernels of wheat kept on increasing in weight up to the period when the percentage of moisture dropped to approximately 40. This period is sometimes spoken of as the period of desiccation. The slight fluctuations on the downward curve are probably due to variations occurring in method of sampling

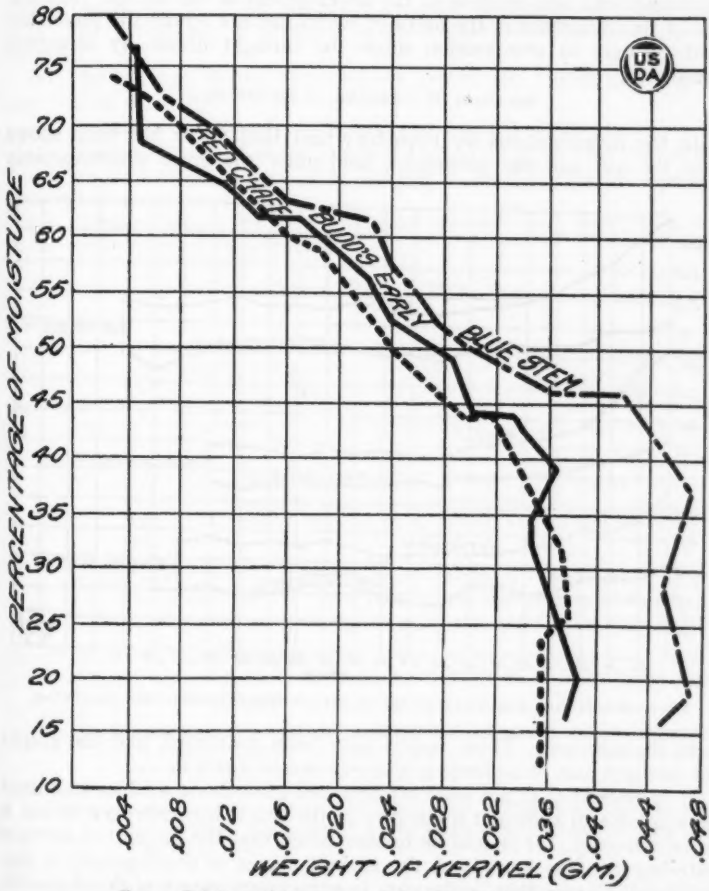

FIG. 1.—Relative moisture content of wheat kernels during growth

which at this period would be more noticeable, because the kernels were heavier, even though the errors were probably not any greater than those during the preceding periods of sampling. Any increase in nitrogen content must precede a period when the kernels contain 40 per cent or more moisture. In the period of embryo development there is considerable moisture in the grain. We also know that in the period of 60 per cent or more of moisture, the simpler forms of nitrogen material are

moving into the kernels at a very rapid rate (*15*) and during the period of decline in moisture content of the grain synthesize into the material known as gluten.

It is contended by Brenchley and Hall (*2*) that in the filling of the endosperm part of the kernel the material is uniform and always possesses the same ratio of nitrogenous to nonnitrogenous material and ash. Thatcher (*33*, *34*,) found that the ratio of protein to carbohydrates decreased with the progress of the development of the kernel. Regardless of this difference in the findings, we should not ignore the possibility that changes in composition might be brought about by abnormal conditions.

RELATION OF NITROGEN TO PHOSPHORUS

In the investigations by Brenchley and Hall (*2*) it has been shown that the ash and the phosphoric acid enter the grain simultaneously

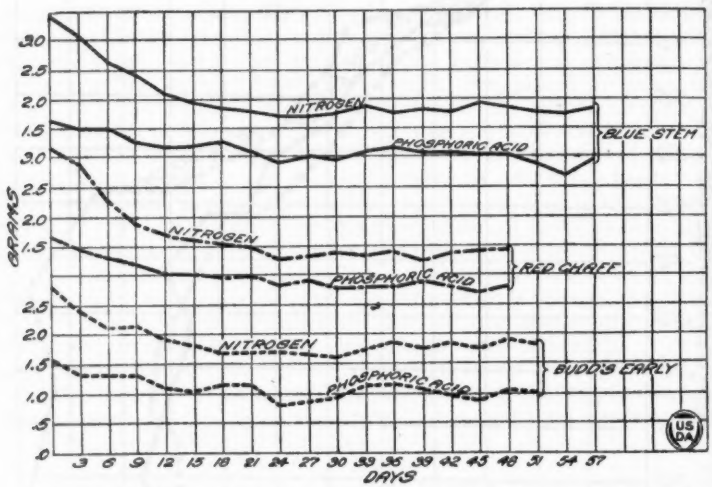

FIG. 2.—Relative time of entrance of phosphoric acid and nitrogen into three varieties of wheat

with the nitrogen. These results have been confirmed, and the graphs for nitrogen and phosphoric acid are shown in figure 2.

It is evident that not only nitrogen but phosphoric acid as well must be available in sufficient quantities in the soil if high-nitrogen wheat is to be obtained. It should be further noted that the amount of nitrogen entering into the kernels at the earliest stages of development is proportionally larger than is the case in subsequent stages of development. The embryo is developed and perfected in this early stage and undoubtedly the larger amount of nitrogen required at this period is for the construction of the embryo. Following the development of the embryo we observe that the phosphoric acid runs parallel with the nitrogen content of the kernel.

SUMMARY

(1) Increasing the distance between the rows seemed to increase the nitrogen content of wheat grown in the nursery under nonirrigated conditions at Pullman.

(2) Hybrid 143 showed a higher percentage of nitrogen when spring-sown than when fall-sown.

(3) Increasing the distance between the rows did not seem to affect the nitrogen content of wheat under irrigated conditions at Grandview.

(4) The percentage of nitrogen in the kernel decreased as the grain matured.

(5) The nitrogen in the plant moved toward the kernel as the grain approached maturity.

(6) Irrigation did not affect the nitrogen content of wheat.

(7) Placing the wheat plant in water moved nitrogen into the kernel.

(8) Wheat kernels showed little increase in weight after the moisture declined to 40 per cent.

(9) Phosphorus entered the grain simultaneously with nitrogen except in the early stages of development.

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RELATIVE SUSCEPTIBILITY OF CITRUS FRUITS AND HYBRIDS TO CLADOSPORIUM CITRI MASSEE[1]

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INTRODUCTION

During the course of citrus-canker investigations by the authors in Alabama, only one other Citrus disease has appeared naturally with any frequency. This disease is citrus scab, caused by *Cladosporium citri* Massee (*5*).[2]

So far as the writers are aware, no one has ever attempted to make any extensive observations to determine the relative susceptibility of Citrus plants to scab. All reports so far published on this subject have been based on occasional field observations of commercial varieties. A summary of the scattered literature reveals the fact that, with a few exceptions, the Citrus plants mentioned as subject to scab by all writers can be arranged into three groups, according to their susceptibility, as follows:

1. Severely attacked.
   - Sour orange.
   - Rough lemon.
   - Lemon.
2. Moderately attacked.
   - Satsuma and other mandarin oranges.
   - Lime (*8, p. 82*).
   - Grapefruit.
   - Trifoliate orange (*2, p. 244*).
3. Rarely attacked.
   - Some few varieties of sweet orange (*1; 7; 6, p. 115*).

Observations on the amount of scab were made by the authors at times during the growing season over a period of four years on a representative lot of Citrus plants growing under approximately the same conditions in the canker isolation field near Loxley, Ala. They were made in connection with the authors' citrus-canker work, accounts of which have appeared (*3, 4*). In these publications, the type, age, and number of plants grown are given in some detail. Owing to the fact that only few of the plants were large enough to set and bear fruit, the degree of susceptibility to scab of the various plants is based on the amount of scab present on the leaves and angular wood.

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[1] Accepted for publication Jan. 29, 1923. Published with the approval of the Director of the Alabama Agricultural Experiment Station. This paper is based on cooperative investigations between the Office of Crop Physiology and Breeding Investigations, Bureau of Plant Industry, United States Department of Agriculture, and the Department of Plant Pathology, Alabama Agricultural Experiment Station.

[2] Reference is made by number (italic) to "Literature cited," p. 959.

RELATIVE SUSCEPTIBILITY TO SCAB

With the exception of *Poncirus trifoliata* (L.) Raf., all the wild relatives of Citrus so far grown in the isolation field are nonsusceptible to scab. With this exception, scab appears to be strictly limited to Citrus fruits and their hybrids. In this respect it differs from citrus-canker, which can attack a large number of Rutaceous plants.

In Table I an attempt has been made to list the majority of Citrus fruits and their hybrids grown in the isolation field, and to indicate the average relative susceptibility of these plants to scab, as has been observed at times over a period of four years under various conditions. The relative susceptibility of the various species and varieties to scab as given in the table is tentative. Many plants are quite susceptible to scab in the early spring when the first growth pushes out, while no scab appears on the foliage of the second or succeeding growths. Some plants, on the other hand, present a more or less scabby appearance during the whole season. Whether the plants placed under the first heading are nonsusceptible in a strict sense, only direct inoculations will show.

TABLE 1.—*Relative susceptibility of Citrus fruits and hybrids to scab*

Genus and species.	Scab not observed.	Slightly susceptible.	Susceptible.	Very susceptible.
Poncirus trifoliata, trifoliate orange		×	×	
Citrus hystrix, round leaf form		×		
C. hystrix, pointed leaf form				×
Citrus sp., Ichang lemon		×		
C. grandis, grapefruit seedlings		×		
Duncan		×		
Sullivan		×		
Shaddock, Fla.				×
Pummelo, Hirado Buntan	×			
Chinese, Orangedale				×
Marks				×
Indian, Roeding			×	
Siamese		×		
C. sinensis, orange, navel	×			
Temple	×			
Japanese No. 1	×			
Japanese No. 2	×			
Sekkan	×			
Pankan	×			
Tankan	×			
C. nobilis, orange, King		×		
C. nobilis var. *deliciosa*, tangerine, Cleopatra		×		
C. nobilis var. *unshiu*, Satsuma			×	
C. mitis, Calamondin				×
Citrus sp. Kanzu				×
Citrus sp. Natsu-mikan	×			
Citrus sp. orange, Naranja				×
Citrus sp. orange, Narute				×
Citrange (*P. trifoliata* × *C. sinenses*)		×	×	
Citrandarin (*P. trifoliata* × *C. nobilis*)		×	×	
Citrunshu (*P. trifoliata* × *C. nobilis* var. *unshiu*)				×
Citrangequat (citrange × *Fortunella margarita*)				×
Citranguma (citrange × *C. nobilis* var. *unshiu*)			×	
Citrangarin (citrange × *C. nobilis* var. *deliciosa*)		×		
Citrangedin (citrange × *C. mitis*)		×	×	
Limequat (*C. aurantifolia* × *F. margarita*)	×			
Bigaraldin (*C. aurantium* × *C. mitis*)	×			
Orangelo (*C. sinensis* × *C. grandis*)	×			
Oranguma (*C. sinensis* × *C. nobilis* var. *unshiu*)	×			
Orangequat (*C. sinensis* × *F. margarita*)		×	×	
Satsumelo (*C. nobilis* var. *unshiu* × *C. grandis*)		×	×	
Clemelo (*C. nobilis* var. *deliciosa* × *C. grandis*)		×		
Siamelo (*C. nobilis* × *C. grandis*)	×			
Tangelo (*C. nobilis* var. *deliciosa* × *C. grandis*)		×		
Siamor (*C. nobilis* × *C. sinensis*)	×			
Sopomaldin (*C. grandis* × *C. mitis*)	×			
Calashu (*C. mitis* × *C. nobilis* var. *unshiu*)		×		
Citraldin (*P. trifoliata* × *C. mitis*)	×			
Calarin (*C. mitis* × *C. nobilis* var. *deliciosa*)	×			
False hybrids		×	×	

Poncirus trifoliata varies from slightly susceptible to susceptible, depending on the season. As a rule, very little scab occurs after primary infection takes place on the unfolding buds and the young shoots in the spring.

The round leaf form of *Citrus hystrix* DC. which is susceptible to canker is only occasionally attacked by scab, while the pointed leaf form which is semi-resistant to canker is very susceptible to scab. Primary infection is usually severe on young leaves and shoots, although the plants present a scabby appearance throughout the growing season.

The fact that *Citrus hystrix*, native of the Philippine Islands, where no scab has ever been found, is susceptible under our Gulf Coast conditions, leads one to assume that *Cladosporium citri* is unable to persist under Philippine conditions, which is one reason why scab has never been reported from the islands, rather than the fact that scab has never been introduced.

The Ichang lemon, possibly a natural hybrid between a lemon and pummelo, appears to be more susceptible to scab than the sour and commercial varieties of lemons.

The behavior of the plants belonging to the *Citrus grandis* group in their susceptibility to scab is quite variable, ranging from nonsusceptible to very susceptible. Whether there is a difference in the varietal resistance is not known. The fact that plants of all ages, conditions, and types were employed makes it doubly hard to draw any conclusions. Judging from observations of other investigators, the grapefruit has only within recent years been reported susceptible to scab. On the whole, it can be said that the ordinary Florida varieties, as represented by Duncan and Sullivan, are only slightly susceptible; the Florida Shaddock, very susceptible; the Indian and Chinese pummelos, susceptible; and the Siamese pummelos, slightly susceptible. The Hirado Buntan pummelo has never developed scab in the field. While there is a decided difference in scab susceptibility of the various plants in the *C. grandis* group, we do not know whether the differences are varietal or due to their individual reaction to environmental conditions which in turn influences scab susceptibility.

No scab has ever been noted on any of the plants belonging to the *Citrus sinensis* group. These observations are in common with those made by most investigators. Stevens (7) reports the Lue Gim Gong as attacked by scab in Florida, and it is the only variety of sweet orange so recorded in America. Earle (1) in Porto Rico finds that some of the round oranges are occasionally attacked by scab. Reinking (6) also reports a plant of the *C. sinensis* group subject to scab in China. On the whole, it can be stated that the sweet orange group, with an occasional exception, is nonsusceptible to scab.

Of the *C. nobilis* group, both the King orange and Satsuma are slightly susceptible to susceptible, depending on weather conditions. Most of the scab occurs during the early spring on the young leaves and small fruits. During late seasons, such as prevailed in 1915 and 1920, Satsumas are badly attacked by scab, resulting in considerable damage to the fruit. The Cleopatra tangerine, which is semiresistant to canker, evidently does not scab. It is one of the few plants promising as canker resistant which is not attacked by scab.

Citrus mitis Blancho is another example of a plant native to the Philippine Islands which is very susceptible to scab under our Gulf Coast conditions. Scab is usually quite severe in the early spring on the

unfolding leaves and twigs. These plants present a more or less scabby appearance throughout the season.

Yuzu or Kansu orange is a plant native of North China; in fact, it grows farther north than any other Citrus. Like *C. mitis*, it is quite resistant to canker, but very susceptible to scab. It is rather peculiar that there are here two plants, one native to the tropics, the other to North China, both somewhat resistant to canker, but susceptible to scab under Gulf Coast conditions. Scab is found on the Kansu plants throughout the growing season.

The *Natsu-Mikan*, possibly a natural hybrid similar to the tangeloes, has so far remained free from scab. The Naranja and Narute oranges, introductions from the Far East, are among the most susceptible plants in the field. These plants present an extremely scabby appearance throughout the growing season.

As a rule, all trifoliate orange hybrids are attacked to some extent in the early spring, varying in intensity, and depending somewhat on the second parent. Thus, citrunshu, with Satsuma as a second parent, is more susceptible to scab than citrange with the common orange as the second parent.

The citrange hybrids vary still more in their susceptibility to scab, ranging from slightly susceptible to very susceptible. The behavior of the citrangequat is extremely interesting. Kumquat, the second parent of this cross belonging to the wild relatives, is outside the range of scab susceptibility. However, when crossed with the citrange, the resulting hybrid is very susceptible to scab, and the plants are generaly scabby throughout the growing season in the field. On the other hand, it is the most promising canker-resistant hybrid that has yet been found.

Limequat has so far remained free from scab. The orangelo and oranguma have also remained clean. The orangequat is a plant similar in many respects to the Satsuma. Thus, in the type of plant and its susceptibility to canker and scab, it behaves like the Satsuma. However, why should it equal the Satsuma in its susceptibility to scab when both of its parents have been so far reported free from scab? Its susceptibility to scab must be closely related to the reaction of this plant to environmental conditions favoring scab attacks.

The bigaraldin presents the opposite extreme in that no scab has been observed on this hybrid, although both its parents, the sour orange and calomondin, are very susceptible to scab.

The mandarin orange-grapefruit hybrids as represented by satsumelo, clemelo, and tangelo vary from slightly susceptible to susceptible. No scab has been observed on siamelo. The hybrids having *Citrus mitis* as one parent either have remained free from scab or are only slightly susceptible, notwithstanding the fact that *C. mitis* is very susceptible to scab.

All the false hybrids vary from slightly susceptible to susceptible, depending on the condition of the plants.

SUMMARY

The range of Citrus plants susceptible to scab has been extended much beyond the few well-known groups of commercial varieties, heretofore reported. No doubt some of the plants reported as nonsusceptible may later be found to be susceptible, while many species and varieties which were not tested will also be found to be susceptible to scab.

All evidences from our observations show that scab is limited to the Citrus fruits and their hybrids, with the exception of *Poncirus trifoliata*. The species and varieties within a group vary in their susceptibility to scab. A good example of this occurs in the *Citrus grandis* or grapefruit group, and is further accentuated among the hybrids. Susceptibility to scab varies not only from season to season, depending on weather conditions, but varies even within the same season. Thus a bad scab year is determined largely by weather conditions just at the time of the formation of the leaves and fruit. In most cases, scab susceptibility narrows down to the reaction of the host plant to environmental conditions, essential to scab infection and the development of the disease. This does not necessarily mean conditions favorable to the best and most normal development of the plants, as under these conditions few of the plants are susceptible to scab. It is extremely interesting to note that in this connection a number of plants which are promising as canker resistant are as a whole susceptible to scab.

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AN IMPROVED METHOD FOR THE DETERMINATION OF NICOTINE IN TOBACCO AND TOBACCO EXTRACTS [1]

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HISTORICAL REVIEW

The two methods which are now more generally used in this country for the estimation of nicotine in tobacco and tobacco extracts are those of Kissling and the silicotungstic acid method as perfected by Chapin.[3] They are the only ones for this determination which have been adopted as official by the Association of Official Agricultural Chemists.[4] It is of interest to note in this connection that while Chapin's procedure for extracts was adopted by the association, a material change appears in the association method. This change is in the amount and manner of adding the alkali preparatory to the distillation; it is opportune, even if made unwittingly, because it tends to increase the accuracy of the method when applied to tobacco. In this connection it is important that a sufficient quantity of alkali be added in the distillation to liberate combined nicotine. The association method should emphasize this point more clearly, since it is possible in following their directions in the distillation to have the solution test alkaline due to free nicotine and yet an insufficient quantity of alkali may have been added to liberate that in combination.

Chapin describes the various procedures which have been proposed for the estimation of nicotine and the defects he found in their application, so it is not necessary to mention them here. Unfortunately, however, Chapin, in his work on the silicotungstic acid method, did not include tobacco in his studies, otherwise he probably would have found that errors may often occur in the use of this method on tobacco, as will be mentioned later. The method as outlined by him was proposed for tobacco extracts and when so applied in the present work has been found to be more satisfactory than for tobacco itself.

While it is generally recognized that the Kissling method will give good results in the hands of an experienced analyst, provided no interfering substances are present in the determination, it is nevertheless, conceded that the silicotungstic acid method will probably give more reliable and concordant results in ordinary use.

After the work reported in this paper was practically completed, the writer learned for the first time, through a mutual acquaintance, that a similar method had been published by Rasmussen,[5] in Denmark. As no reference to it could be found in the literature at hand, a copy was obtained

[1] Accepted for publication Jan. 22, 1923. Published by permission of the Director of the Kentucky Agricultural Experiment Station.

[2] The author desires to thank Dr. A. M. Peter, Head of the Department of Chemistry, for helpful criticisms offered during the progress of this investigation.

[3] CHAPIN, R. M. THE DETERMINATION OF NICOTINE IN NICOTINE SOLUTIONS AND TOBACCO EXTRACTS. *In* U. S. Dept. Agr. Bur. Anim. Indus. Bul. 133, p. 21. 1911.

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and it was found that while Rasmussen's method is somewhat similar in principle, it is entirely different in technic from the one described in this paper. As no determinations by the former method have been made here, its accuracy is not known. Certain features of it, however, are mentioned for comparison with the writer's procedure.

The extraction of nicotine from the tobacco to which alcoholic soda has been previously added is carried on in a flask containing a mixture of equal parts of ethyl ether and petroleum ether, by frequent shaking for 5 hours. The solvent is then filtered from the tobacco through a folded filter covered with a glass plate. An aliquot is taken for acid extraction. With extracts, 3 or 4 gm. of the sample are placed in a 100 cc. flask together with 5 cc. concentrated caustic soda, 5 cc. water, and 50 cc. of the above mixed ethers. The mixture is allowed to stand 4 or 5 hours with frequent shaking, then 25 cc. of the solvent is drawn off and treated as before. In both cases the nicotine is precipitated with silicotungstic acid. Doubts might be raised as to the complete extraction of the nicotine, especially from extracts handled in the manner indicated, as compared with a Soxhlet extraction of the sample as prepared for analysis here. Furthermore, errors may arise due to evaporation when the solvents are filtered and aliquots withdrawn in the manner described. Finally, no provision is made for the prevention of emulsions often obtained in the acid washing of the ether extract of some samples and without which no method of this character is practical.

EXPERIMENTAL WORK

The writer, having occasion to determine the nicotine content of a large number of various grades and varieties of tobacco grown in Kentucky, found that it would be very convenient to have a method which would be more rapid than either of the official methods and equally accurate. As several determinations were to be made at the same time and facilities for making simultaneous steam distillations were not at hand, it was desirable to have a method that would, if possible, eliminate such distillations. A steam distillation of nicotine is objectionable inasmuch as it is often a long, tedious procedure that requires careful attention in order that the boiling liquid shall be maintained at a low volume to remove all nicotine.

The writer's previous experience with the Kissling method has shown that there are two principal errors to be avoided: First, the presence of ammonia in the nicotine distillate, due to its incomplete separation from the ether extract of the sample, and, second, the possible loss of nicotine in the evaporation of this ether extract previous to its distillation. The first error will, of course, give high results, due to the ammonia being titrated and calculated as nicotine, whereas the other causes low results; consequently, satisfactory figures obtained by the method may be sometimes the result of a balancing of errors. On the other hand, experience with the silicotungstic acid method in this work has shown that while it is decidedly preferable to the Kissling, nevertheless certain points in its use have to be carefully supervised in order to obtain satisfactory results. The writer's experiments show that this method as outlined by Chapin gives more satisfactory results on nicotine extracts than on tobacco. The reasons for this will be mentioned later.

Experience here has shown that the distillation as prescribed in the silicotungstic acid method, even though carried on under proper condi-

tions, is sometimes a long, tedious procedure; and, if not so conducted, will often give inaccurate results on tobacco. Accordingly, it was thought possible to substitute an ether extraction of the sample for the steam distillation of it in the silicotungstic acid method, in order to obtain the nicotine for precipitation. Afterwards, the nicotine could be extracted from the ether by acid and determined in the manner prescribed in this method. Accordingly, considerable work has been carried on with this in view, and, after many preliminary determinations, a method has been devised which has usually been found to be more rapid than either of the official methods and, with some samples at least, gives more accurate results than the silicotungstic acid method unless the latter is carried on under carefully controlled conditions. It combines the good features of both official methods and eliminates an undesirable one common to both, namely, the steam distillation. The method is as follows:

REAGENTS

Alcoholic sodium hydroxid solution: Dissolve 6 gm. of sodium hydroxid in 40 cc. of water and 60 cc. of 90 per cent alcohol.

Silicotungstic acid solution: Prepare a 12 per cent solution of the silicotungstic acid having the formula $4H_2O$, SiO_2, $12WO_3$, $22H_2O$. (There are several silicotungstic acids. The acids $4H_2O$, SiO_2, $10WO_3$, $3H_2O$ and $4H_2O$, SiO_2, $12WO_3$, $20H_2O$, do not give crystalline precipitates with nicotine and should not be used.)

Ethyl ether of quality equal to U. S. P. concentrated.

Dilute hydrochloric acids (1 to 4) and (1 to 1,000).

DETERMINATION

Weigh 1 gm. of tobacco extract (high grade 40 per cent) or more of extracts containing less nicotine; or 5 gm. of finely powdered tobacco which has been previously dried at room temperature or slightly above, to permit powdering, and put into a beaker or, preferably, a porcelain dish. Add 2.5 cc. of the alcoholic sodium hydroxid to the tobacco powder or about 5 cc. to the extract; it is necessary that enough alcoholic sodium hydroxid be added to liberate the nicotine. Follow in the case of extracts with a sufficient quantity of pure powdered calcium carbonate to form a moist but not lumpy mass. Mix thoroughly with a pestle or spatula, transfer to a suitable container in a Soxhlet extractor and exhaust for about 5 hours with ether. (Probably another solvent could be substituted for this purpose.) It is important that the temperature of the cooling water during the extraction should not be much over 20° C., and sufficient solvent should be used in order that its volume in the extraction flask should never be less than about 25 cc. After the extraction is made, the exhausted sample is removed and the excess of ether recovered, but the volume of ether extract should not be permitted to go below the above minimum or more if necessary to keep the extractive matter in solution at ordinary temperature. (If the temperature of the cooling water is too high and the volume of solvent in the flask becomes too low during the extraction, there may be a small loss of nicotine from samples of high percentage.)

The ether extract is transferred to a separatory funnel, preferably with a short stem, the extraction flask washed with a little ether and finally about twice with hydrochloric acid (1 to 4) to remove all nicotine.

The extract is shaken with four to six portions (10 cc. each) of cold dilute hydrochloric acid (1 to 4). If an emulsion forms during the successive extractions with the acid, this can be prevented by adding a small amount of 95 per cent alcohol, about 1 cc. being sufficient for each separate extraction. (When it is found necessary to use alcohol, it is essential that it be added after the shaking has been completed. Further shaking should be avoided as much as possible, as one or two inversions of the funnel will break the emulsion. A minimum amount of alcohol and shaking are best, since it has been found that when alcohol is added along with the acid, followed by vigorous shaking, it has some solvent action on extraneous material that precipitates with the reagent. Consequently, alcohol should not be used unless necessary, and only in the manner described.) The successive extractions are run through a funnel into a 100 cc. graduated flask. A small ball of fine glass wool may be placed in the neck of the funnel. The extraction should be continued until a few drops of the acid solution give no turbidity with the reagent. The funnel is carefully washed with water and the volume made to 100 cc. An aliquot corresponding to about 0.25 gm. or more of extract or 1 to 2 gm. of tobacco, depending on the amount of nicotine present, is placed in a beaker and diluted to 400 to 500 cc. volume with distilled water. A sufficient quantity of dilute hydrochloric acid (1 to 4) is then added so as to have at least 3 cc. or more present, to each 100 cc. of liquid, including the quantity of acid contained in the aliquot. At this point, the solution should test distinctly acid with a few drops of methyl orange; if not acid, more should be added. There is then added, with constant stirring, 1 cc. of the silicotungstic acid reagent for each 0.01 gm. of nicotine supposed to be present. Stir thoroughly for about 3 to 5 minutes or until the precipitate is crystalline and settles quickly. To insure an excess of the reagent, a few drops are added to the clear supernatant liquid and the absence of any precipitate noted. The solution is allowed to stand at least 18 or preferably about 24 hours. The precipitate is filtered on an ashless filter (C. S. & S. No. 590 or similar quality) and preferably through a Hirsch or Buchner funnel by decanting first the clear liquid and testing a few drops of it with a little nicotine solution to insure an excess of reagent. By decanting practically all the clear solution the filtration is more quickly made. (In case of very small precipitates, the addition of a small amount of ashless filter paper pulp to the precipitate at the time it is to be filtered will insure a clear filtrate.) When the precipitate is transferred to the filter, if the filtrate should be turbid, it is filtered again until clear. If, at the beginning, the precipitate is made crystalline by proper stirring, the filtration is made quickly and without trouble. The precipitate is thoroughly washed with cold dilute hydrochloric acid (1 to 1,000). Transfer the precipitate, without removing from the paper, to a weighed platinum crucible, dry carefully at low heat until the paper is charred, then ignite over a Bunsen burner until all carbon is eliminated. Finally, heat over a Teclu or Meker burner for not over 10 minutes, or over a moderate blast for about 5 minutes. It is not advisable to prolong the final heating beyond the time mentioned, because of a very small but continued loss at this temperature. The weight of the residue multiplied by 0.114 gives the weight of nicotine present in the aliquot used.

In the development of this method, the following experiments were made to test important features of the work:

ABSENCE OF FOREIGN MATERIAL IN THE ACID SOLUTION OBTAINED FROM THE ETHER EXTRACT WHICH MIGHT PRECIPITATE WITH THE REAGENT

As experiments had shown that such material would more likely be obtained from tobacco than from extracts, aliquots of the same acid solution of tobacco were compared. One aliquot was distilled and determined by the silicotungstic acid method while the other aliquot was handled at this stage as described in the method above. The results are given in Table 1.

TABLE I.—*Percentage of nicotine in tobacco (moisture free)*

No.	Chapin's silicotungstic acid method.	Improved method.
61422	1. 29	1. 33
61423	2. 17	2. 23
61426	0. 70	0. 75
61436	7. 26	7. 30
61437	5. 47	5. 51
Average	3. 38	3. 42

It is apparent from Table I that in the acid solution obtained from the ether extract of these samples, no foreign material is present which might precipitate with the reagent and materially affect the results, unless it is volatile with steam.

THE AMOUNT OF ALCOHOL USED IN THE MANNER PRESCRIBED IN THE METHOD APPARENTLY DOES NOT HAVE ANY APPRECIABLE DETRIMENTAL EFFECT

Duplicate determinations were made on different tobaccos, in one of which alcohol was used as prescribed, while in the other it was omitted. The results are given in Table II.

TABLE II.—*Percentage of nicotine in tobacco (moisture free)*

No.	Alcohol used.	No alcohol used.
80236	6. 94	6. 80
80237	5. 98	6. 02
80238	6. 35	6. 30
80239	5. 72	5. 82
80273	2. 98	2. 87
80274	2. 10	2. 05
80275	1. 79	1. 77
80276	0. 97	0. 9
80382	7. 77	7. 7
80383	7. 04	6. 94
80384	5. 78	5. 70
Average	4. 86	4. 82

Apparently alcohol used in the quantity and manner prescribed in the method has very little, if any, effect on the results. The use of alcohol would be more likely to affect the results in the analysis of tobacco than in that of extracts. Experience has shown that it is not usually required for extracts and is not always necessary for tobacco.

Alcohol has been found, however, to have a solvent action for extraneous matter contained in the ether extract of some tobaccos if it is added with the acid and thoroughly shaken, as is done in the acid extraction. For example, two of the samples in Table II, when alcohol was used in this manner, gave the following results: No. 80236 = 7.37 per cent and No. 80237 = 6.32 per cent nicotine. On the other hand, it has been repeatedly found that when as much as 5 cc. alcohol is added to an aliquot after the acid extraction is made, identical results are obtained as compared with another aliquot containing no alcohol. However, it should not be used except where it is necessary and then only in the manner prescribed.

When a comparison was made of this method with Chapin's silicotungstic acid method, it was found that higher, and in some samples of tobacco abnormally higher, results were obtained by the latter method. Much better agreement, however, was shown with extracts. Steam distillations showed that whereas not over 750 cc. of distillate was usually sufficient to obtain the nicotine from extracts, a much larger volume was generally required for tobacco, notwithstanding the fact that a smaller amount of nicotine might be present in the larger distillate. Again, it was found essential that the solution in the distilling flask should be maintained at about 15 to 25 cc. in order to distill over the nicotine in a minimum volume of distillate. If the volume of liquid greatly exceeds this extreme difficulty is met with in distilling the nicotine, especially from tobacco. For example, tests with negative results have been made for nicotine in a distillate coming from a comparatively large solution, although further concentration to the optimum volume showed its presence. Another interesting fact observed in the steam distillation was that the nicotine results obtained by its use, especially with tobacco, were largely influenced by the amount of alkali used in the distillation. For instance, it was found that where 2 gm. of sodium hydroxid was used in the distillation higher results were always obtained than when smaller amounts were employed, despite the fact that in the former case the nicotine usually came over in a smaller volume of distillate. In all distillations the liquid in the distilling flask was alkaline, and sufficient alkali was present to liberate combined nicotine. It has been shown in this connection that the larger amount of alkali liberates some volatile compound other than nicotine which comes over in the distillate and is precipitated by the reagent. This substance occurs in variable quantity in tobacco, and a distillate containing it generally has some odor and color. The compound does not give the characteristic test for nicotine, but forms with silicotungstic acid a dirty white precipitate which becomes pinkish on standing. Where this substance occurs in appreciable quantity it can be obtained by distilling with an excess of alkali (2 gm.) the exhausted tobacco obtained from the ether extraction, or, after a weak alkaline distillation is completed, a further addition of alkali up to the above amount and continued distillation will show its presence. There is no doubt but that the excess of alkali and heat used in the distillation acts to a greater or less extent on the tobacco residue

contained in the small volume necessary to liberate the nicotine, and forms a volatile product or products which are found in the distillate, precipitate with the reagent and affect the results. Further work will be done to determine, if possible, the nature of this reaction.

The following precautions are therefore necessary in the distillation: (1) Use the minimum amount of alkali necessary to liberate all the nicotine; (2) keep the volume of liquid in the flask at about 15 to 25 cc. to facilitate the distillation of the nicotine; (3) continue the distillation until a few cubic centimeters of the distillate shows no opalescence when treated with a drop of dilute hydrochloric acid and a drop of the silicotungstic acid. Under such conditions, however, a large volume of distillate will sometimes be obtained, as shown in Table III.

TABLE III.—*Percentage of nicotine in tobacco (moisture free); effect of different amounts of NaOH in distillation of 5 gm. sample on the volume of distillate necessary to liberate the nicotine.*

No.	2 gm. NaOH.	Volume of distillate.	Final test for nicotine.	Less than 2 gm. NaOH.	Volume of distillate.	Final test for nicotine.
61469	7.55	900	Absent.	[1] 7.38	1,500	Absent.
				[1] 7.29	1,500	Do.
				[2] 6.90	2,900	Do.
61537	6.33	900	do.	[2] 5.67	1,800	Present.
				5.98	2,400	Absent.
80073	7.85	1,800	do.	[1] 6.76	1,000	Do.
	7.83	1,800	do.	[1] 6.65	1,000	Do.
80144				[2] 7.32	2,100	Present.
				[2] 7.51	2,700	Absent.

[1] NaOH used in distillation, 0.25 gm.
[2] NaOH used in distillation, 0.15 gm.

DIRECT PRECIPITATION OF NICOTINE

It was thought that it might be possible with some extracts, provided no interfering substances were present, to make a direct precipitation of the nicotine and thereby eliminate the distillation and ether extraction necessary in the other methods. Experiments were made, therefore, by taking a weighed sample of extract, diluting it to a definite volume and directly precipitating the nicotine in an aliquot with the reagent in the manner prescribed in the improved method as to volume, acidity, and handling of the precipitate. While the precipitates were often colored, very satisfactory results were obtained on most of the extracts, although precipitable matter in others proved to be detrimental. The results obtained are given in Table V.

This method was also tried on tobacco by making an aqueous or weak akaline extraction, filtering and using an aliquot as described in the preceding paragraph. It was not found applicable, however, as shown by the determinations below, on two samples from Table III. No. 61469 gave 9.13 per cent nicotine by an akaline extraction and No. 61537 gave 7.53 per cent by an akaline and 7.37 per cent nicotine by an aqueous extraction.

The results obtained by the improved method on tobacco and extracts in comparison with Chapin's silicotungstic acid method, together with those found by the latter when variable amounts of alkali were used in the distillation, are given in Tables IV and V.

TABLE IV.—*Percentage of nicotine in tobacco (moisture free).*

No.	Chapin's silicotungstic acid method.		Improved method.
	2 gm. NaOH used in steam distillation of 5 gm. sample.	Less than 2 gm NaOH used in steam distillation of 5 gm. sample.	
61469	7.55	[1] 7.38 [1] 7.29	6.99 7.01
		[2] 6.90	7.00
61537	6.33	7.19 [2] 5.98	5.98 6.18
			6.08
80073	7.85 7.83	[1] 6.76	6.70 6.88
	7.84	[1] 6.65	6.79
		6.71	
80074	6.37	[1] 6.19	6.01
80076	5.32	[1] 5.09	4.92
80117	3.67	.....	3.37
80121	3.97	.....	3.68
80126	2.71	.....	2.48
80129	4.57	.....	4.43
80130	4.50	.....	4.24
80134	1.75	.....	1.55
80144	.....	[2] 7.51	7.67
80195	.....	[2] 7.77	7.83
80263	.....	[1] 2.56	2.57 2.58
			2.58

[1] NaOH used in distillation, 0.25 gm.
[2] NaOH used in distillation, 0.15 gm.

TABLE V.—*Percentage of nicotine in tobacco extracts and products*

No.	Brand.	Guaranty.	Direct precipitation method.	Chapin's silico-tungstic acid method.[1]	Improved method.
43889[2]		16.42	18.77	17.31	15.96
				[3] 17.44	16.71
				17.38	16.34
80054	"Nico-Fume"	40.00	41.60	40.78	40.77
80055	"Hammond's Tobacco Extract"	4.00	4.26	4.27	4.25
80056	"Nikoteen"	30.00	31.67	30.85	30.78
80064	"Thompson's Rose-Nicotine"	5.00	5.13	5.18	[4] 4.89
				[5] 5.26	[4] 4.89
				5.22	4.89
80065		.18	.72	.13	.13
80066		3.57	4.78	3.70	[4] 3.51
				[5] 3.82	[4] 3.51
				3.76	3.51
80356	"Hall's Nicotine Solution"	40.00	40.43	[3] 40.62	39.69
80357	"Hall's Nicotine Sulfate"	40.00	40.15	[3] 39.82	39.88
40358	"Hall's Nicotine Fumigator"	12.50	.....	[3] 13.26	13.18
80823	"Black Leaf 40"	40.00	40.54	40.00	40.13
80824		.....	1.64	[6] 2.47	1.65
				2.50	
				2.49	
80826		.....	94.63	[7] 93.73	93.42
			94.70	[7] 93.75	93.60
				[7] 94.60	94.14
			94.67		
				94.03	93.72
80828		40.00	40.61	[8] 39.74	40.39
			40.73	[8] 40.19	40.88
			40.67	39.97	40.64
80829	"Black Leaf 40"	40.00	42.21	[8] 41.87	42.09

[1] In this method, the following weights of sample were distilled with 2 gm. NaOH unless otherwise indicated:
Samples containing 50 per cent or more of nicotine, 0.6 gm.
Samples containing 30 to 50 per cent of nicotine, 1 gm.
Samples containing 3 to 30 per cent of nicotine, 2 gm.
Samples containing less than 3 per cent of nicotine, 5 gm.

[2] This extract had been prepared for several years and was supposed to contain 16.42 per cent of nicotine, 2.78 per cent of pyridin and 2.37 per cent of ammonium chlorid. The nicotine guaranty is probably only approximate. The results obtained for nicotine by all methods include any pyridin that may have been present in the washed precipitate.

[3] NaOH used in distillation, 0.60 gm.

[4] Duplicates from same ether extraction.

[5] NaOH used in distillation, 0.15 gm.

[6] NaOH used in distillation, 0.24 gm.

[7] NaOH used in distillation, 0.90 gm.

[8] NaOH used in distillation, 0.30 gm.

SUMMARY

(1) A method has been devised for the determination of nicotine in tobacco and tobacco extracts which has been found to be as rapid and as accurate as the silicotungstic acid method and more satisfactory on tobacco. The other standard method commonly used for nicotine is the Kissling.

(2) The new procedure eliminates an undesirable feature, namely, the steam distillation, common to both of the above methods.

(3) It has been shown that special precautions should be taken in carrying on the distillation in the silicotungstic acid method. Under proper conditions very good results were usually obtained by its use; otherwise, serious errors were often found when working on tobacco.

(4) A direct precipitation method which obviates a preliminary ether extraction or steam distillation to obtain the nicotine, has also been tried and fairly satisfactory results were obtained on most of the tobacco extracts employed. It was not found, however, to be applicable to tobacco and a few extracts.

NUTRITIVE VALUE OF MIXTURES OF PROTEINS FROM CORN AND VARIOUS CONCENTRATES[1]

By D. BREESE JONES, *Chemist in Charge;* A. J. FINKS, *formerly Associate Biological Chemist;* and CARL O. JOHNS, *formerly Chemist in Charge, Protein Investigations Laboratory, Bureau of Chemistry, United States Department of Agriculture*

INTRODUCTION

The nutritive value of mixtures of peanut and soy-bean flours as supplements to wheat-flour proteins has been shown in previous publications (*4, 5*) [2] from this laboratory. This paper is a continuation of these studies with whole yellow corn. In addition to the peanut and soy-bean flours as protein supplements to corn are also included meal made from tomato-seed press cake and coconut meal made by both the solvent and expression processes. Data on both the chemical and nutritional studies of the proteins from tomato seed and coconut have already been published (*3, 6, 7, 8, 9*). Each of the concentrates mentioned has been found adequate for the normal growth of albino rats as the sole source of protein in otherwise complete diets.

It has been shown by several investigators that whole corn is not adequate for normal growth. Feeding experiments with zein, a protein which constitutes more than one-half of the total proteins in maize, have shown it to be deficient in lysine and tryptophane—amino acids which are essential for the growth of animals. Experiments also have been recorded which show that when corn proteins are fed at a high enough intake level—as, for example, in corn gluten—normal growth can be secured.

Whole corn constitutes a large part of the ration of many animals and it is of practical importance to the feeder to know whether the addition of small quantities of protein concentrates such as coconut meal, tomato-seed, press cake, soy-bean and peanut flour will result in a protein mixture which will meet the nutritional requirements for normal growth. Our experiments with these concentrates show that a mixture of 25 parts of tomato-seed press cake, soy-bean flour, or peanut flour with 75 parts of corn satisfies the protein requirement for the normal growth of white rats. In the case of the coconut meal, equal parts were necessary to secure the same result. This may not have been due, however, to inferiority in the nutritional value of the coconut-meal proteins, but rather to the bulky character of the meal caused by its higher content of crude fiber. Chemical studies of the proteins of these concentrates have shown that they are relatively high in lysine and tryptophane, and are therefore well suited to supplement the corn proteins which are deficient in these amino acids.

EXPERIMENTS WITH WHOLE YELLOW CORN MEAL

Two diets containing 7.2 per cent of protein in which the proteins came solely from whole yellow corn were used. Diet 1 contained 16 per cent of butter fat. Diet 2 was made up of the same composition, with

[1] Accepted for publication Nov. 24, 1922.
[2] Reference is made by number (italic) to "Literature cited," p. 977–978.

the exception that lard was used in the place of butter fat. The growth curves in Chart 1 show that about equally poor growths resulted with both of these diets, an average gain in weight of only 0.73 gm. per gram of protein intake being obtained. Although diet 2 contained no source of vitamin A other than the yellow corn (unless the lard may have contained some), growth was obtained at approximately the same rate as when diet 1 with the butter fat was used. No indication of xerophthalmia was observed. These facts indicate the presence of vitamin A

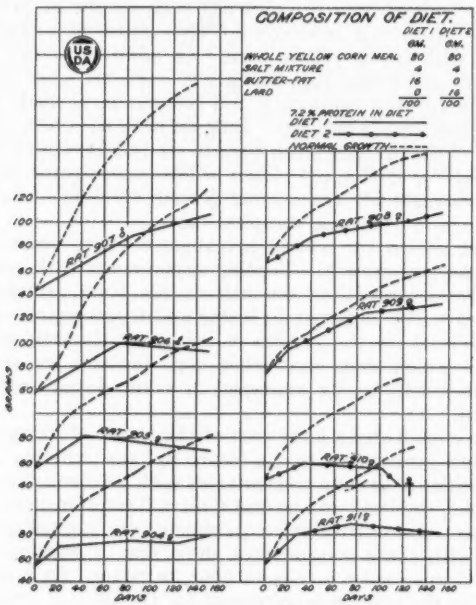

CHART 1.—Curves showing the poor rate of growth of young albino rats fed on a diet the proteins of which came solely from whole yellow corn.

in yellow corn, as has previously been shown by Steenbock and Boutwell (*13*).

EXPERIMENTS WITH CORN AND TOMATO-SEED PRESS CAKE

Studies made in this laboratory (*8*) have shown that tomato-seed press cake contains nearly 37 per cent of protein and that the nutritionally essential amino acids are well represented in these proteins, being relatively high in arginine, lysine, and cystine. Qualitative tests also show the presence of tryptophane. The nutritional adequacy of these proteins, as shown by chemical studies, has been confirmed by feeding experiments (*3*).

On a diet in which the proteins were furnished by 75 parts of whole yellow corn meal and 25 parts of tomato-seed press cake, growth at the normal rate was secured (Chart 2). On such a diet, the tomato-seed press cake furnished 7.4 per cent, and the corn 5.4 per cent of protein.

Decreasing the protein level to 7.2 per cent, but keeping the proportion of tomato-seed press cake to corn the same, resulted in a rate of growth somewhat below the normal. This was better, however, than the diet in which corn alone was used to furnish the same amount of protein. An average gain in weight of 0.94 gm. per gram of protein intake was obtained during an 11-week period on this mixture. With the corn alone, an average gain of only 0.73 gm. was secured. The low level of the protein intake was responsible for a rate of growth somewhat below normal. By using a diet somewhat below the minimum requirement of protein for normal growth, the maximum gain in weight per gram of protein intake is obtained. Such a diet affords a better basis for a comparative study of the growth-promoting value of different proteins.

EXPERIMENTS WITH CORN AND COCONUT PRESS CAKE

Diets in which the proteins were obtained from mixtures of equal parts of coconut meal and whole yellow corn enabled albino rats to

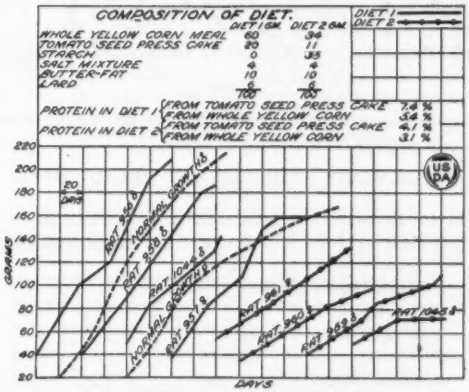

CHART 2.—The plain continuous lines represent the rate of growth of rats on diet 1, containing 5.4 per cent of protein from corn, supplemented with 7.4 per cent of protein from tomato-seed press cake; the growth represented by the continuous dotted lines was made on diet 2, of which 3.1 per cent of the proteins came from corn, and 4.1 per cent from tomato-seed press cake.

grow at the normal rate. On such a diet, the coconut meal furnished 8.4 and the corn 3.6 per cent protein, respectively. This experiment shows that the coconut proteins supplement corn proteins, since better than normal growth was obtained with 12 per cent of the mixture of proteins, while 13.1 per cent of coconut proteins alone, in an otherwise adequate diet, just barely sufficed for normal growth (7). When coconut meal furnished 4.2 per cent and corn 5.4 per cent protein in the diet, growth was somewhat subnormal. The coconut meal used in these diets was made both by the expression process (A) and by the solvent process (B). The processes seemed to be of equal value as regards the nutritive properties of the meal produced. The results of this experiment are recorded in Chart 3. The efficiency of a coconut-corn protein mixture for promoting growth, as shown by these experiments, is in agreement with the results reported by Maynard and Fronda (11), who found that "a mixture of corn meal protein and coconut-oil-meal protein was of

slightly higher quality than the former alone, but much better than the latter alone."

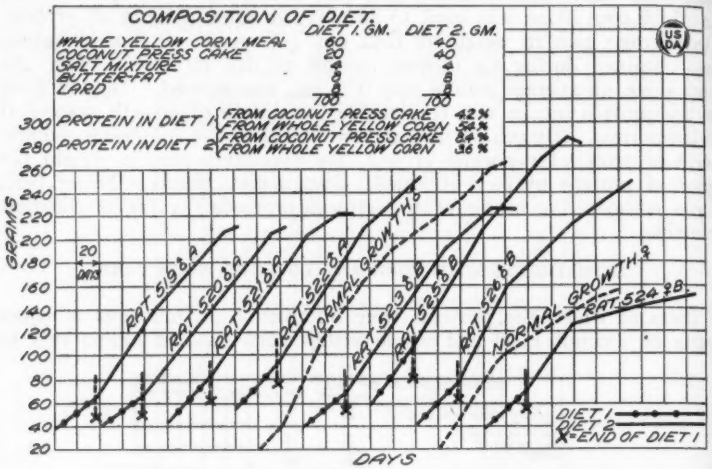

CHART 3.—Curves showing the growth-promoting efficiency of a coconut-corn protein mixture. The compositions of the diets are given on the chart.

EXPERIMENTS WITH CORN AND PEANUT MEAL

When peanut meal forms the sole source of protein at intake levels of from 15 to 18 per cent in diets which are otherwise complete, Daniels and

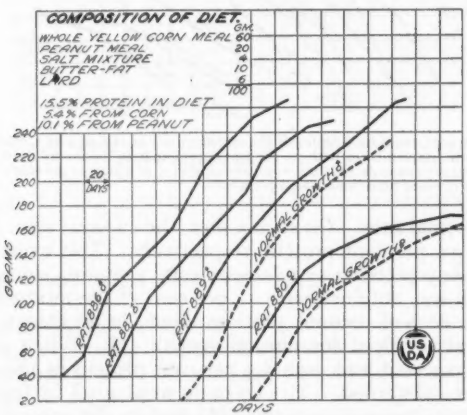

CHART 4.—These curves represent the growth-promoting value of a protein mixture, 5.4 per cent of which was furnished by corn meal, and 10.1 per cent by peanut meal.

Loughlin (*1*) have shown that normal growth can be secured. In our experiments normal growth was obtained with mixtures consisting of 75 parts of corn meal and 25 parts of peanut flour at a protein level of 15.5 per cent. From such a protein mixture two-thirds of the protein was

furnished by the peanut flour (Chart 4.) By keeping the proportion of the concentrate to corn the same, but lowering the protein level of the diet to 7.2 per cent, a better rate of growth was obtained than when corn

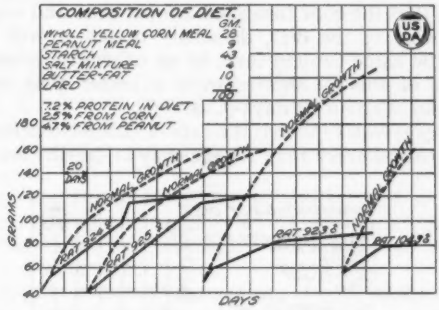

CHART 5.—Growth curves showing the supplementary value of peanut proteins when fed with corn proteins, at a low protein intake level.

alone furnished the protein in a diet otherwise adequate at the same level (Chart 5).

The average gain in weight per gram of protein in this mixture during an eleven-week period was 1.23 gm. compared with 0.73 gm. on corn alone. Rat 1043, in the above lot, was not included in the average.

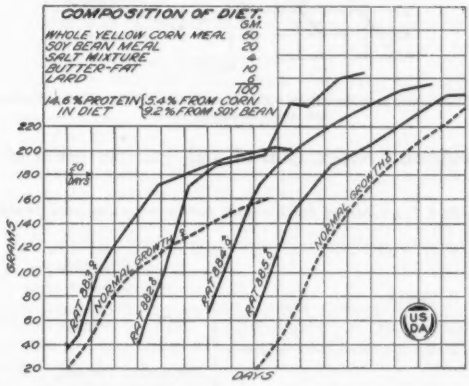

CHART 6.—These curves show the excellent growths obtained on a diet 5.4 per cent of the proteins of which was furnished by corn, and 9.2 per cent by soy-bean meal.

EXPERIMENTS WITH SOY BEAN AND CORN

It has been shown (*12, 2*) that soy-bean proteins are adequate for the normal growth of albino rats. Our experiments were made with a diet in which 25 parts of soy-bean flour replaced an equal quantity of corn meal. This diet contained 14.6 per cent of protein. Better growth than at the normal rate was obtained (Chart 6) when to such a mixture was added a suitable inorganic salt mixture, lard and butter fat. In this diet, 9.2 per cent of the protein was furnished by soy beans and 5.4 per

cent by the corn. McCollum (*10*) has shown that a supplementary relationship exists between corn and soy-bean proteins in the ratio of 6 per cent of the former to 3 per cent of the latter.

When the protein level of the corn-soy-bean mixture was reduced to 7.2 per cent, so that the corn furnished 2.6 per cent and soy bean 4.6 per cent of the protein of the diet, decidedly better growth resulted than when corn at the same protein level in an otherwise adequate diet was used. Rat 926 of this lot showed even a better than normal rate of growth at the end of about 95 days (Chart 7).

During an eleven-week period, the rats of this lot (excluding rat 1041, which for some reason other than a deficiency in the diet made practically

CHART 7.—Curves showing the supplementary value of soy-bean proteins when fed with corn proteins, at a low protein intake level.

no growth) made an average gain of 1.48 gm. per gram of protein consumed.

TABLE I.—*Gain of body weight per gram of ingested protein*

Diet.[1]	Protein in—			Gain per gram (11 weeks).
	Corn.	Concentrate.	Total.	
	Per cent.	*Per cent.*	*Per cent.*	*Gm.*
Corn	7.2	.....	7.2	0.73
Corn+tomato-seed press cake	3.1	4.1	7.2	.94
Corn+peanut meal	2.5	4.7	7.2	1.23
Corn+soy-bean meal	2.6	4.6	7.2	1.48

[1] These diets were made adequate with respect to the nutritionally essential factors other than proteins. (See charts.)

Although the ratio of the percentage of concentrate protein to corn protein is not exactly the same in these diets (Table 1), it can safely be concluded that the growth-promoting value of the proteins of these concentrates, as supplements to the proteins of corn, is in the following order: Soy bean, peanut, and tomato seed. In the experiments with the coco-

nut press cake, no diets containing as low a protein content as 7.2 per cent were used. Therefore, a direct comparison of the nutritive value of the proteins of this concentrate with the other concentrates studied, cannot easily be made.

SUMMARY

Mixtures consisting of 25 parts of tomato-seed press cake, soy-bean flour, or peanut flour, and 75 parts of yellow corn meal, which contained from 12 to 15 per cent of protein, have been found to furnish proteins adequate for the normal growth of albino rats when incorporated in a diet made nutritionally adequate with respect to the dietary factors other than protein.

A mixture of equal parts of corn meal and coconut meal at a protein level of 12 per cent was found efficient for growth at the normal rate. The growth was somewhat subnormal when the proportion of protein was reduced to 9.6 per cent.

From experiments in which the mixtures of corn meal and concentrates furnished 7.2 per cent of protein, it is concluded, from the gain in weight per gram of protein consumed, that the comparative growth-promoting value of the proteins of tomato seed, peanut and soy bean, as a supplement to corn proteins, is in the order: Soy bean, peanut, and tomato seed.

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